THE MULTIFUNCTIONAL PROTEIN BETA-CATENIN AND PANCREATIC ORGANOGENESIS

by

Brett Kenneth Baumgartner

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Brett Kenneth Baumgartner**

has been approved by the following supervisory committee members:

Charles Murtaugh	, Chair	**07/28/2014** Date Approved
Richard Dorsky	, Member	**07/28/2014** Date Approved
Suzanne Monsour	, Member	**07/28/2014** Date Approved
Gabrielle Kardon	, Member	**07/28/2014** Date Approved
Mark Metzstein	, Member	**07/28/2014** Date Approved

and by **Lynn Jorde** , Chair/Dean of

the Department/College/School of **Human Genetics**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

During pancreatic organogenesis, all mature cell types in the pancreas arise from common multipotent pancreatic progenitor cells (MPCs), localized to the distal tip of the expanding epithelium. The dysfunction or destruction of pancreatic β-cells results in diabetes. Endeavors to treat this disease by replacing β-cells with new cells generated from stem cells will benefit from exploiting the pathways involved in the normal development of this pancreatic cell type from MPCs. Prior studies indicated that the protein β-catenin was required for the development of exocrine acinar cells, but the precise role of β-catenin in endocrine cell development remained controversial. β-catenin also performs two separate functions, one as a crucial component of canonical Wnt signaling and the other at cell-cell junctions where it binds with E-cadherin to stabilize adherens junctions. The purpose of this dissertation is to understand and separate the specific roles of β-catenin, whether signaling or structural in nature during pancreas development and β-cell development, particularly as it relates to the growth and maintenance of MPCs. Following the elimination of all β-catenin function in the early pancreas, I found that β-catenin is not required for the differentiation of β-cells, but that it was required to establish β-cell mass. This requirement played out not in β-cells themselves, but at the level of normally specified MPCs, in which β-catenin is necessary to both expand and maintain distal patterning in progenitors, providing a sufficient number of progenitors capable of producing β-cells. To determine whether

the observed growth and patterning defects were due to the signaling or structural role of β-catenin, I use a signaling-deficient yet structurally-competent form of the protein. I found that the growth of the pancreas is dependent on the signaling role of β-catenin and surprisingly that patterning and exocrine acinar cell development require the structural role of β-catenin. Thus, both the signaling and structural roles of β-catenin are necessary for separate but equally important aspects of pancreas development. Together, the information presented in this dissertation provides new molecular insights into the role of β-catenin during pancreas and β-cell development.

This dissertation is dedicated to my family, past, present, and future

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CHAPTER 1

INTRODUCTION

The prevalence of pancreatic diseases in the human population, chiefly diabetes and pancreatic cancer, serves as incentive for developing treatments based on the understanding of pancreas biology and development. The multifunctional pancreatic epithelium executes both endocrine and exocrine tasks through specific functional domains. The exocrine domain, which makes up the majority of pancreatic mass, consists of acinar cells and a highly branched ductal network that function together to generate and transport digestive enzymes into the intestine to assist digestion. Pancreatic adenocarcinoma (PDAC), a particularly deadly cancer, arises from within the exocrine pancreas. The endocrine domain comprises clusters of cells named the islets of Langerhans which contain several cell types. The primary function of these islet cells is to regulate blood glucose through the action of the majority cell type, insulin secreting beta-cells, and their less abundant counterparts, glucagon secreting alpha-cells. Other cell types exist in islets, albeit fewer in number than either beta or alpha cells, namely somatostatin producing δ-cells, ghrelin producing ε-cells, and pancreatic polypeptide producing PP cells. The autoimmune destruction or dysfunction of β-cells leads to diabetes, a prevalent and growing disease worldwide.

Despite significant morphological, functional, and pathological differences, all pancreatic cells arise from common multipotent pancreatic progenitor cells (MPCs)[1,2]. The specification, maintenance, and expansion of these MPCs is crucial to the proper formation of the adult pancreas, as shown by the development of a smaller and dysfunctional organ following early partial ablation of progenitors[3]. Critical aspects of the instructional program that directs MPC specification, maintenance, self-renewal, and finally fate decision throughout pancreatic organogenesis and homeostasis will be addressed in this thesis. A special emphasis will be placed on the endocrine pancreas and the role of the signaling and structural protein β-catenin during pancreas development, with an end-goal of understanding β-cell development for the purpose of generating cell-based therapies for diabetics.

Diabetes

Diabetes mellitus can be characterized as a metabolic disease caused by reduced insulin functionality that results in high blood glucose leading to serious health concerns. As a disease family, diabetes can be etiologically subdivided into type 1 and type 2 diabetes (T1DM and T2DM, respectively). Although both T2DM and T1DM are both the result of elevated blood glucose, the causes of these two subforms differ greatly. In type 1 diabetes, autoimmune destruction of β-cells results in insulin insufficiency, leading to elevated blood sugar that usually manifests in juveniles. In contrast, TD2M is not a disease of the pancreas per se, but rather stems from insulin resistance in skeletal muscle and adipose tissue, with strong correlation to obese and aged individuals. The inability of these nonpancreatic tissues to recognize insulin signals leads to the inability to uptake

glucose and subsequent elevated blood glucose. Eventually, T2DM leads to insulin deficiency and reduced β-cell mass, likely due to added stress placed on β-cells to secrete more insulin to resistant tissues[4,5].

Even though the origin and causes for each of these diseases differ, both forms of diabetes are increasing dramatically in prevalence. In the United States alone, due to unspecified reasons, T1DM increased by 23% from 2001 to 2009, with approximately 3 million people currently living with the disease. An even more disturbing trend exists in relation to T2DM in which over 10% of people over 20 years old have diabetes with another 80 million people being considered prediabetic. As a whole, diabetes cost $245 billion dollars in 2012 and diabetics on average spent over two times the amount of non-diabetics on healthcare [6,7]. Moreover, persons living with diabetes must constantly monitor blood glucose, and must often supply exogenous insulin in order to properly maintain glucose homeostasis. Therefore, developing more cost-effective and more permanent treatment options for diabetics is of significant importance. β-cell replacement has been proposed as a potentially curative treatment; however, obtaining sufficient β-cells poses a significant challenge.

Sources of β-cells

To date, the only successful β-cell replacement therapies involve transplantation of cadaver tissue, either isolated islets or whole pancreas. Whole pancreas transplantation is a relatively new procedure with success rates comparable to other organ transplants. In fact, most T1DM transplant recipients are no longer insulin-dependent for several years following surgery [8]. However, whole pancreas transplants are complicated

and are only performed in combination with kidney transplantation in diabetics with end-stage renal disease. Furthermore, immunosuppressant therapies must be employed to avoid organ rejection, leading to weakened immune response. As an alternative to whole pancreas transplants, islets can be isolated from whole pancreata and successfully transplanted into T1DM patients by following a procedure known as the Edmonton protocol [9]. Although less invasive than whole organ replacement, obtaining cadaver islets is not a trivial process and in fact, 3-4 donor organs must be harvested in order to obtain sufficient numbers for a single transplantation. While outcomes are nearly 100% efficacious within the first year, islet function decreases in subsequent years, often necessitating another round of islet transplantation[10,11]. The short-term success of transplantation combined with the difficultly of obtaining sufficient quality islets provides incentive to identify potential nondonor sources of β-cells, including self-replication, neogenesis, and in vitro differentiation.

Perhaps the most straightforward source of new β-cells is to induce pre-existing β-cells to proliferate. Justifying this approach are studies indicating that self-replication is the main driver of expansion and regeneration of β-cells following depletion or during instances of high metabolic demand such as obesity and during pregnancy [12-15]. Several molecules and mechanisms have been identified that can encourage and regulate β-cell proliferation; however, the efficacy of each molecule is dependent upon specific genetic or physiological conditions and do not ubiquitously increase β-cell mass [5]. Moreover, in T1DM, β-cell mass is dramatically reduced and the lack of starting material may not allow for sufficient β-cell self-replication, necessitating other sources of starting material.

The adult pancreas exhibits phenotype plasticity between differentiated cell types. This is well demonstrated in pancreatic cancer, where exocrine acinar cells undergo metaplasia turning into duct-like cells that later give rise to cancerous lesions [16]. The same plasticity that allows for such transformation could be harnessed to reprogram other pancreatic cell types or adult pancreatic progenitors into β-cells. Acinar cells, partly because they are directly derived from multipotent progenitor cells (MPCs), and partly because they are the most abundant cell type in the pancreas, represent a large population with the potential to become β-cells, but do so only after having been transduced with β-cell-specific transcription factors [17]. Glucagon secreting α-cells have also been shown to reprogram into β-cells, after the ectopic expression of the β-cell-specific transcription factor Pax4 [18] and after near complete β-cell ablation [19]. Regardless of the source of cells that might be reprogrammed into β-cells, the ability to induce such transformation without dramatic external manipulation is not possible at present.

A major challenge of β-cell replacement via transplantation, replication, or neogenesis is whether any of these methods can efficiently produce sufficient numbers of β-cells to maintain glucose homeostasis. The in vitro generation of β-cells from pluripotent stem cells (embryonic stem cells or induced pluripotent stem cells) serves as a potential solution to this problem, providing, in essence, an endless supply of cells for transplantation. Essential transcription factors needed for β-cell development have been identified; however, introducing foreign DNA constructs to drive expression of these transcription factors carries with it unknown consequences, including potential disruption of other essential genes. Current pluripotent stem cell differentiation protocols involve step-wise differentiation first into definitive endoderm, then foregut, followed by

pancreatic fate and finally, hormone-producing endocrine cells [20]. Importantly, these protocols do not involve the introduction of DNA-constructs, but rather rely on precise temporal administration of recombinant signaling proteins [21]. However, even though functional glucose-sensing β-cells cells differentiate in vivo from the engraftment of pancreatic endocrine progenitors, in vitro differentiated β-cells often lack the ability to sense and respond to glucose [20,22]. These advances suggest that deriving β-cells from stem cells is indeed possible. Nevertheless, there are many facets of in vivo β-cell differentiation and maturation that remain poorly characterized, preventing the development of such cell-based treatments. The signaling pathways and interactions that guide pancreas development from progenitor through functional mature pancreatic cells remain particularly elusive. Further study of in vivo pancreas development is necessary to enhance efforts to drive stem cell differentiation into β-cells.

Mouse pancreas development

In mice, the first morphological evidence of pancreas development occurs around embryonic day 9.5 (E9.5), when evaginating ridges of foregut endoderm emerge bi-directionally into pancreatic mesenchyme to form a pair of dorsal and ventral pancreatic buds. Preceding this morphological change is the expression of the transcription factor pancreatic and duodenal homeobox 1 (*Pdx1)* at ~E8.5. Based on the almost complete pancreatic agenesis observed in its absence, *Pdx1* was the first gene discovered as a master regulator of pancreas development [2,23-26]. Shortly after specification, all pancreatic progenitors (MPCs) express *Pdx1*, and a different but equally crucial transcription factor, pancreas transcription factor 1-a (*Ptf1a)* [27]. *Ptf1a* further specifies

pancreatic fate over other endodermal fates in *Pdx1* is expressing cells [28], and is likewise essential for proper pancreas development and expressed by MPCs [29]. These two transcription factors together help instigate the pancreas developmental program, and lineage tracing experiments show that all pancreatic cell types arise through Pdx1 and Ptf1a expressing progenitors. [1,28,30,31]. Following specification, during a period called the primary transition, concomitant with high levels of proliferation and the appearance of endocrine α-cells, the mouse pancreatic epithelium undergoes a major morphological change where pancreatic buds spatially stratify into two distinct domains, distal "tips" and proximal "trunks", a process henceforth referred to as patterning. The newly patterned epithelium expands into the pancreatic mesenchyme, which secretes signaling factors necessary for growth, patterning, and allocation of progenitors into differentiated fates [2,32] (Fig. 1.1). The distal tips contain MPCs, while proximal trunks contain MPC derived bi-potent progenitors that give rise to endocrine islets and duct cells. MPCs produce all pancreatic lineages until approximately the beginning of the secondary transition at E13.5, after which distal MPCs are restricted to an acinar fate [1]. Bi-potent progenitors, on the other hand, produce both ducts and islets throughout in utero development [33]. The decision between duct and islet is marked by the expression of the transcription factor neurogenin-3 (Ngn3), which is expressed throughout in utero development by endocrine precursors [30,34,35]. Importantly, trunk cells that express high levels of Ngn3 comprise a nondividing, endocrine-committed precursor population that directly differentiates into endocrine cells, after which they regain proliferative abilities [2]. Careful attention has been paid to the transcriptional networks involved in the

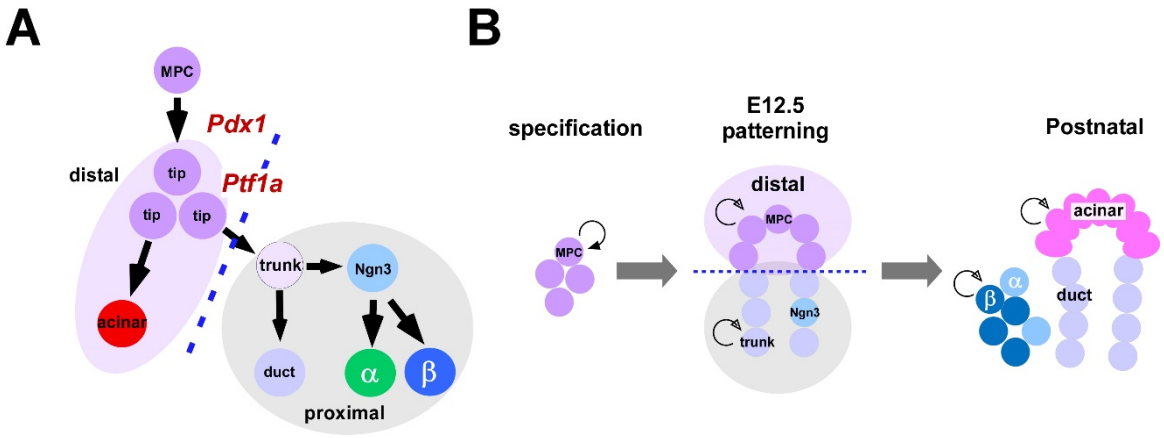

Figure 1.1: Pancreas lineages and development. Pancreas lineages and development are depicted schematically. (A) All pancreatic lineages arise from MPCs that express the transcription factor Pdx1. The division between Ptf1a+ distal and proximal domains is represented by the blue dashed line. Proximal trunks give rise to ducts and Ngn3+ endocrine precursors. (B) Cartoon schematic showing the morphology and stages of pancreas development. The black arrows indicate proliferation. The division between distal and proximal is represented by the blue dashed line. The semicircular arrows represent proliferation.

differentiation of MPCs to all other pancreatic cell types [2]; however, the upstream signaling events that control these transcriptional changes remain less understood.

Signaling pathways and pancreas development

As signaling pathways mediate communication between cells and responses to the cellular environment, manipulating them could potentially affect stem cell differentiation extrinsically. Nevertheless, signal transduction pathways must act in a strict spatiotemporal fashion to bring about various aspects of organogenesis. Several signaling pathways, including Notch, Wnt, Fibroblast Growth Factor (FGF), and retinoic acid (RA), participate in pancreas development [2]. Perturbations in the Notch signaling pathway illustrate critical context-specific control of early pancreas development and lineage allocation. Early studies of the pathway associated Notch signaling as an inhibitor of pancreatic endocrine differentiation [36,37] and later studies further demonstrated that Notch also inhibited acinar cell development [38-40]. These observations support recent reports that advocate a role for Notch in modulating distal-proximal patterning, wherein Notch promotes proximal/bipotent fate over distal/MPC fate, and duct over endocrine fate in bipotent trunk progenitors [41-43]. However, Notch is but one of many signaling pathways involved in pancreas development, and a major gap exists in understanding how multiple signaling pathways communicate to bring about the complex morphological and fate decisions of pancreatic organogenesis. Chapter 2 of this thesis specifically addresses the interaction of Notch signaling and β-catenin, a nonredundant component of canonical Wnt signaling and the ultimate focus of this thesis.

Wnt/β-catenin signaling

First described as wingless signaling in *Drosophila* [44], Wnt signaling is involved in the normal development of most metazoan tissues, and perturbations in the pathway contribute to human diseases, possibly including pancreatic cancer and diabetes [45]. Wnt ligands are secreted and bind to specific receptor combinations to activate signaling. Traditionally, Wnt signaling is divided into canonical and noncanonical signaling pathways, which require specific Wnt ligand and receptor combinations. In the absence of a Wnt ligand, cytoplasmic β-catenin is degraded by a complex partly consisting of APC, Axin, and the kinase GSK3. The phosphorylation of β-catenin by GSK3 results in the ubiquitinization of β-catenin and proteosomal destruction. Following Wnt ligand binding to LRP5/6 and Frizzled receptors, the protein Disheveled is activated, which causes complex disassembly, release of β-catenin, and accumulation of unphosphorylated β-catenin in the cytoplasm. When the levels of accumulation reach an unknown threshold, some β-catenin enters the nucleus where it binds to specific members of the Tcf/Lef family of transcription factors, displacing transcriptional repressors and recruiting transcriptional co-activators eventually culminating in the transcription of specific target genes [45].

Study of vertebrate Wnt signaling has been hampered by the redundancy signaling elements including Wnt ligands (19 in mice) and Frizzled receptors (10 in mice). However, the canonical Wnt signaling pathway requires β-catenin regardless of the many ligand-receptor combinations that may exist, and perturbations of this single molecule allow for more straightforward pathway manipulation in the Wnt receiving cell[45,46]. Likewise, in Wnt sending cells, eliminating the function of the Wnt modifying

proteins *Porcn* or *Evi* effectively prevents the secretion of Wnt ligands[47]. Thus, abolition of Wnt/β-catenin signaling can be accomplished by either deleting β-catenin in receiving cells, or in Wnt secreting cells through the deletion of Porcn or Evi.

Wnt/β-catenin in the pancreas

Wnt/β-catenin signaling is required for the expansion, differentiation, and maintenance but not for the specification of the pancreatic epithelium[46,48]. The purpose of this thesis is to uncover the mechanisms through which β-catenin instructs progenitor differentiation into the various pancreatic lineages. During early development of the pancreas in mice, *Xenopus*, and zebrafish, activation of the Wnt pathway appears to prevent pancreas development at the level of specification, by pushing early specified pancreatic progenitors away from a pancreatic fate toward other endodermal fates [46,49]. In mice, this is highlighted by a study that demonstrated that prior to E11.5, activation of β-catenin with an early acting *Pdx1-Cre* transgene (*Pdx1-Cre*^{early}) led to severe pancreatic hypoplasia, whereas activation with the later-acting "*Pdx1-Cre*^{late}" transgene led to the opposite phenotype, a hyperplastic organ, mainly due to increased acinar cell number [48].

In contrast to the early activation of Wnt/β-catenin signaling, inhibition of Wnt pathway activity through the expression of a dominant-negative Frizzled8 Wnt receptor did not disrupt pancreas specification, but produced an overall smaller organ [50]. Prior studies using *Pdx1-Cre*^{early} transgenes to delete β-catenin from the early embryonic pancreas found mutant pancreata to be significantly smaller than littermate controls and severely deprived of acinar cells; nevertheless, a qualitatively normal number of

endocrine β- and α-cells were present [51,52]. Moreover, somewhat similar acinar hypoplasia was observed after deleting the Wnt target gene c-Myc [53,54], supporting a Wnt/β-catenin-dependent pancreatic growth model. Conversely, following the deletion of β-catenin using the *Pdx1-Cre^{late}* transgene [48], which acts several days later than *Pdx1-Cre^{early}*, acinar cells developed normally but islet cell number was slightly decreased [55], suggesting that β-catenin was required by endocrine but not exocrine cells for growth and expansion later in development. These seemingly conflicting reports are likely the result of the temporal efficacy of Cre-mediated recombination in the two different strains [46]. Moreover, during the postnatal period and during regeneration from pancreatitis-induced injury, β-catenin is required for acinar cell proliferation [56]. Whether Wnt/β-catenin signaling is required for endocrine cell development and expansion remains somewhat controversial: where some data suggest it is required for the expansion of endocrine cells [57,58], a separate study indicates that it is dispensable for β-cell proliferation [56]. Therefore, the precise roles of β-catenin during pancreas development, particularly as it relates to establishing endocrine β-cell mass, remain to be unraveled. Chapter 2 of this thesis specifically addresses this question through extensive spatiotemporal analysis of β-catenin deficient pancreata.

β-catenin as a structural protein

The signaling function of β-catenin was first described in Drosophila by the identification of its homolog *armadillo*, a component of wingless signaling [59]. During the same period, β-catenin was biochemically isolated, together with α-catenin and γ-catenin/plakoglobin, as a binding partner for E-cadherin, and the cadherin-catenin

complex was proposed to bridge the actin cytoskeleton of adjacent cells to stabilize and polarize epithelial sheets [60,61]. These two distinct roles for β-catenin/armadillo may control different aspects of cellular behavior as shown in studies in both Drosophila and mice [61-63]. In pancreas development, changes to polarity and actin dynamics lead to precise morphological rearrangements that are necessary for epithelial patterning [64] and mutations in actin remodeling proteins can lead to defects in epithelial organization and cell differentiation [65-67]. Thus, a structural role for β-catenin is plausible in the pancreas, yet separating the structural and signaling functions of β-catenin has been challenging in this organ and elsewhere because Tcf/Lef and transcriptional co-activator binding domains overlap with the E-cadherin and α-catenin binding domains (Fig. 1.2). The central region of the β-catenin protein, made up of several armadillo repeats, is required for both signaling and structural functions, while the flanking N and C-terminal domains are required to recruit transcriptional co-activators [68-71].

Even though obvious considerations must be paid to potential defects in cadherin-binding/structural integrity in studies eliminating β-catenin function, most have concentrated only on its role in Wnt signaling. For example, numerous mouse studies investigating tissue-specific Cre-mediated deletion of β-catenin have been mainly focused on its signaling role [72]. Recently, through the introduction of two separate mutations based on binding domain characterization, Konrad Basler and his colleagues created a Wnt signaling-deficient, yet structurally-competent form of β-catenin ($\beta CAT^{double\text{-}mutant}$ or βCAT^{DM}). This important study demonstrated that the signaling and structural roles of β-catenin were both essential for neural tube development [63], a previously unknown and underappreciated finding. In Chapter 3 of this thesis, I will

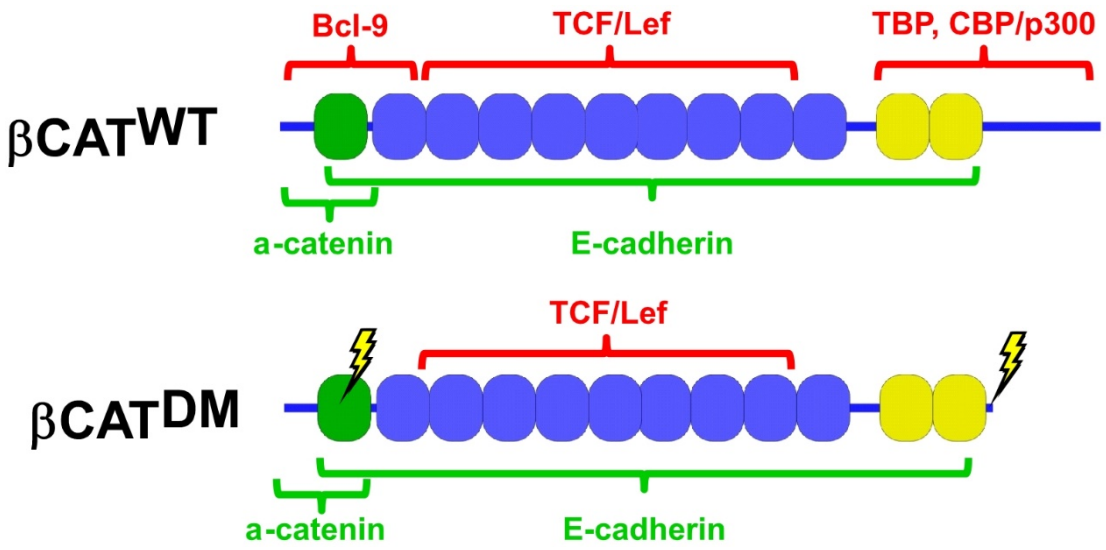

Figure 1.2: The signaling and structural binding domains of β-catenin. β-catenin comprises an N-terminus, 12 armadillo repeats (colored boxes), and a C-terminal domain. (A) Wildtype β-catenin (βCAT^{WT}) binds to transcriptional co-activators at the N- and C-terminus of β-catenin, and to Tcf/Lef in the internal armadillo repeat region (blue squares). β-catenin binds to E-cadherin in the armadillo repeats and to α-catenin at the N-terminal side of the armadillo repeat region (green square). (B) βCAT^{DM} mutants are truncated at the C-terminus and mutated D164A at the N-terminus (yellow lightning bolts) and no-longer bind transcriptional co-activators(red lettering), but still retain E-cadherin and α-catenin binding domains.

demonstrate that the signaling and structural functions of β-catenin also control different aspects of pancreas development.

Summary

The loss of functional β-cells or response to insulin leads to diabetes, and the number of both type 1 and type 2 diabetics is dramatically increasing worldwide. Replacing lost or dysfunctional β-cells through transplantation, self-replication, or neogenesis from existing acinar tissue is limited by the efficiency of each method to restore adequate β-cells to restore glucose homeostasis. In vitro differentiation of β-cells from stem or progenitor cells could provide a potentially endless supply of functional cells. However, due to a lack of understanding of the in vivo mechanisms involved during their development, no fully functional β-cells have been generated in vitro. Therefore, a better understanding of the signaling and downstream transcriptional changes that guide the expansion and differentiation of pancreatic progenitors is needed.

Multipotent pancreatic progenitors (MPCs) are capable of giving rise to all pancreatic lineages through the early phases of development [1] and the number of MPCs determines the final size of both the exocrine and endocrine pancreas. However, unlike acinar cells, endocrine cells do not directly arise from MPCs; rather, they arise via MPC-derived bipotent progenitors. Each progenitor pool, MPCs or their bipotent offspring, occupies a specific niche within the pancreatic epithelium, with MPCs being found at the distal tip and bipotent cells found in proximal trunks. Maintaining this distal-proximal patterning along with their ability to self-renew is crucial to the development and final volume of pancreatic β-cells.

Previous studies indicate that the signaling and structural protein β-catenin is necessary for the embryonic differentiation, postnatal growth, and adult regeneration of exocrine acinar cells but not for pancreatic endocrine cells [51,52,55]. Nonetheless, the precise role of β-catenin in the regulation of MPCs and in other pancreatic lineages remains unknown and in the case of β-cells, remains controversial. Potentially complicating such studies is the fact that β-catenin is a multifunctional protein, participating in canonical Wnt signaling pathway as well as constituting a structural component of E-cadherin junctions. The work presented in this thesis centers on the roles of β-catenin during early mouse pancreas development. I specifically address the roles of β-catenin in maintaining distal-proximal patterning and progenitor expansion and, through the use of unique genetic tools, dissect the signaling and structural roles of the protein.

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CHAPTER 2

DISTINCT REQUIRMENTS FOR BETA-CATENIN IN PANCREATIC EPITHELIAL GROWTH AND PATTERNING

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Distinct requirements for beta-catenin in pancreatic epithelial growth and patterning

Brett K. Baumgartner, Gabriela Cash, Hillary Hansen, Shawn Ostler, L. Charles Murtaugh *

University of Utah, Department of Human Genetics, 15 N. 2030 E. Rm 2100, Salt Lake City, UT 84112, USA

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ABSTRACT

Pancreatic exocrine and endocrine lineages arise from multipotent pancreatic progenitor cells (MPCs). Exploiting the mechanisms that govern expansion and differentiation of these cells could enhance efforts to generate β-cells from stem cells. Although our prior work indicates that the canonical Wnt signaling component β-catenin is required qualitatively for exocrine acinar but not endocrine development, precisely how this requirement plays out at the level of MPCs and their lineage-restricted progeny is unknown. In addition, the contribution of β-catenin function to β-cell development remains controversial. To resolve the potential roles of β-catenin in development of MPCs and β-cells, we generated pancreas- and pre-endocrine-specific β-catenin knockout mice. Pancreas-specific loss of β-catenin produced not only a dramatic reduction in acinar cell numbers, but also a significant reduction in β-cell mass. The loss of β-cells is due not to a defect in the differentiation of endocrine precursors, but instead correlates with an early and specific loss of MPCs. In turn, this reflects a novel role for β-catenin in maintaining proximal–distal patterning of the early epithelium, such that distal MPCs resort to a proximal, endocrine-competent "trunk" fate when β-catenin is deleted. Moreover, β-catenin maintains proximal–distal patterning, in part, by inhibiting Notch signaling. Subsequently, β-catenin is required for proliferation of both distal and proximal cells, driving overall organ growth. In distinguishing two distinct roles for β-catenin along the route of β-cell development, we suggest that temporally appropriate positive and negative manipulation of this molecule could enhance expansion and differentiation of stem cell-derived MPCs.

Introduction

The adult pancreas can be divided into two functionally distinct domains, the exocrine function constituted by enzyme-secreting acinar cells and ducts, and the endocrine function performed by the islets of Langerhans. The division between the two domains begins shortly after specification where the pancreatic epithelium is separated into a distal "tip" domain at the periphery of the organ, which contains multipotent pancreatic progenitors (MPCs), and a proximal trunk domain, which contains bipotent progenitors that give rise to islet and duct cells. The capability of MPCs to produce all pancreatic lineages lasts roughly until the secondary transition at E13.5, after which distal tips are restricted to an acinar fate (Pan and Wright, 2011; Zhou et al., 2007). The early specification, maintenance and self-renewal of embryonic progenitors is critical to the development of the adult organ, as evidenced by the generation of a dysfunctional and smaller pancreas when early progenitors are partially ablated (Stanger et al., 2007).

With an ultimate goal of generating β-cells from stem cells, much effort has gone into understanding the transcription factors and intercellular signals that control pancreas progenitor patterning, expansion and differentiation (Pan and Wright, 2011). As signal transduction pathways mediate the response of progenitors to their microenvironment, they might serve as facile targets for manipulating stem cell differentiation. Nonetheless, while metazoan development requires only a few core signaling pathways, these can be deployed in complex and changing ways during organogenesis. This is exemplified, in the pancreas, by recent studies of the Notch signaling pathway. Originally implicated specifically as a negative regulator of endocrine differentiation (Apelqvist et al., 1999; Jensen et al., 2000), Notch has since been shown to inhibit acinar development as well (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003), potentially through modulating proximal–distal patterning of the early organ (Afelik et al., 2012; Magenheim et al., 2011; Schaffer et al., 2010). Although much has been gleaned from studying Notch and other pathways individually, relatively little is known about these signals coalesce to pattern and specify progenitors in the developing pancreas.

The goal of the present study is to elucidate the cellular mechanisms by which β-catenin, a component of the Wnt signaling pathway, controls the allocation of endocrine and exocrine lineages from pancreatic progenitor cells. In previous studies using

* Corresponding author.
E-mail address: murtaugh@genetics.utah.edu (L.C. Murtaugh).

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two different "*Pdx1-Cre*^{early}" transgenic mice, both driving recombination in the early embryonic pancreas, conditional β-catenin knockout pancreata were found to be drastically smaller than wild-type and largely devoid of exocrine acinar cells, while containing a qualitatively normal complement of endocrine α- and β-cells (Murtaugh et al., 2005; Wells et al., 2007). However, deleting β-catenin using a different "*Pdx1-Cre*^{late}" transgene, beginning several days later in development (Heiser et al., 2006), produced little effect on acinar cell development but caused a mild decrease in islet cell mass (Dessimoz et al., 2005). It was hypothesized in that study that β-catenin was required separately for proliferation or survival of endocrine cells and exocrine progenitors. The discordant results of these studies may reflect the efficiency and timing of the different *Pdx1-Cre* deletor strains used (Murtaugh, 2008).The requirement for β-catenin in acinar cells persists through adulthood, when it is necessary for steady-state turnover and acinar cell regeneration following injury (Keefe et al., 2012). The role of Wnt/β-catenin signaling in differentiated endocrine cells has been an area of debate, with some studies suggesting it promotes β-cell proliferation and function (Dabernat et al., 2009; Rulifson et al., 2007), and another indicating that it is dispensable for adult mouse β-cell proliferation (Keefe et al., 2012). Together, these studies suggest that the contribution of β-catenin to endocrine islet development remains to be unraveled. Using time- and lineage-specific deletion experiments, we sought to investigate the roles of β-catenin during embryonic pancreas development, particularly in establishing endocrine β-cell mass.

Materials and methods

Mice

All experiments were performed according to protocols approved by the University of Utah IACUC. We obtained several mouse strains from the Jackson Laboratory: floxed and germline β-catenin loss-of-function mice (*Ctnnb1*$^{tm2Kem/J}$ and *Ctnnb1*$^{tm2.1Kem}$, henceforth *Ctnnb1*^{lox} and *Ctnnb1*$^{\Delta}$, respectively) (Brault et al., 2001); *Ngn3-Cre* BAC transgenic mice (Schonhoff et al., 2004) and the Cre-dependent EYFP reporter strain *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ (Srinivas et al., 2001), henceforth *R26R*^{EYFP}. *Pdx1-Cre* and *Pdx1-CreERT* transgenic mice (Gu et al., 2002) were provided by Doug Melton (Harvard University). To induce recombination with the *Pdx1-CreERT* transgene, we administered tamoxifen (Sigma) suspended in corn oil (Sigma) to pregnant dams, typically 8–16 weeks of age, by oral gavage. Embryos were genotyped by PCR, using primer sets described previously (Gu et al., 2002; Murtaugh et al., 2005).

Tissue processing and staining

Pregnant dams were euthanized with isoflurane followed by cervical dislocation. Whole embryos (E13.5 and younger) and pancreata (E14.5 and later) were dissected in ice-cold PBS for processing. Tissues were fixed overnight at room-temperature with zinc-buffered formalin (Polysciences) for paraffin sections or with 4% paraformaldehyde/PBS (2 h-overnight at 4 °C for frozen sections, and further processed as previously (Keefe et al., 2012; Kopinke and Murtaugh, 2010; Murtaugh et al., 2005)). A series of duplicate paraffin sections (6 μm) were collected sequentially across multiple slides, spaced with skipping to span the entire pancreas in the following age-dependent manner: for E17.5, 10 slides with 180 μm between sections; E12.5–E14.5, 8 slides with 96 μm between sections; E11.5, 7 slides with 84 μm between sections on slides. In this way, the entire volume of each pancreas is sample on multiple individual slides. Similarly, frozen sections (8 μm) were collected serially over 6–10 slides such that the each slide contained representative sections throughout the organ. For labeling S-phase nuclei, mice were injected with BrdU (50 μg/g body weight) one hour prior to sacrifice.

Antibodies used for immunostaining are listed in Table S1, and all secondary antibodies (raised in donkey) were purchased from Jackson Immunoresearch. Immunostaining was performed as previously (Keefe et al., 2012; Kopinke and Murtaugh, 2010; Murtaugh et al., 2005), including high-temperature antigen retrieval for paraffin sections. For anti-BrdU staining, frozen sections were pre-treated with DNase I (700 u/μl, in 40 mM Tris–HCl pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$) at room temperature for 30 min (Ye et al., 2007). Bright field images were obtained using an Olympus CX41 microscope and MicroSuite software. For immunofluorescence, Fluoromount-G (Southern Biotech) was used as a mounting substrate and images were obtained using an Olympus IX71 microscope and MicroSuite software. Identical exposure times and post-processing adjustments performed in Adobe Photoshop were used across control and experimental genotypes.

Quantification and analysis

To measure β-cell mass, pancreas size, and volume or number of cells expressing various markers, serial sections were stained by immunohistochemistry, and all sections on a single slide were photographed individually (4× original magnification at E17.5, and 10–20× for all other time points), to provide a representation of the entire pancreas. ImageJ (NIH) software was used to measure the surface area occupied by stained tissue. For Ptf1a, c-Myc, and Ngn3 at E11.5 and E12.5, stained nuclei were counted in Adobe Photoshop. The total number of cells per pancreas (E11.5–E13.5) was estimated by multiplying the number of cells counted per slide by the number of slides in the series. Calculations, graphs and *P*-values (two-tailed, unpaired *t*-test) were generated in Microsoft Excel, and results are presented as mean ± s.e.m.

For lineage tracing experiments using *Pdx1-CreERT* and *R26R*^{EYFP}, we photographed several independent fields (20× original magnification), per embryo, across multiple pancreatic sections per slide. For acinar cell labeling indices, we used the additive image overlay feature of ImageJ (NIH) software to identify the overlap of DAPI, amylase (acinar cells) and EYFP, and counted cells using the Analyze Particles function (Kopinke and Murtaugh, 2010). For all other differentiation markers, cells were counted using the Count Tool in Adobe Photoshop.

Explant cultures and wholemount immunostaining

For ex vivo explant cultures, the dorsal buds of E11.5 pancreata were dissected in ice-cold sterile PBS, and cultured at the air-media interface on 0.4 μm pore size PTFE cell culture inserts (Millipore), in DMEM with 10% fetal bovine serum and antibiotics. A small piece of tissue was collected for genotyping purposes at the time of dissection. Explants were treated with 100 nM of the γ-secretase inhibitor DBZ (Millipore 565789) for 3 days, while controls received no treatment. Media was changed daily.

Wholemount immunofluorescence was performed as previously described (Kopinke and Murtaugh, 2010). Briefly, explants were fixed overnight in 4% PFA, washed and stored in methanol until staining. For staining, explants were rehydrated to PBS, permeabilized for 1 h with 1% Triton-X100 in PBS, and then placed in blocking solution (5% donkey serum and 0.3% Triton X-100) for 2 h. Primary and secondary antibody incubations were performed overnight at room temperature. Explants were cleared in BABB (2:1 benzyl alcohol:benzyl benzoate) prior to imaging. Confocal images were obtained using an Olympus FV-1000 microscope.

Results

Both pancreas size and beta-cell mass depend on β-catenin function in multipotent progenitor cells

As in our previous study (Murtaugh et al., 2005), we used the *Pdx1-Cre* early transgene (henceforth, *Pdx1-Cre*) (Gu et al., 2002; Heiser et al., 2006) to delete the conditional *Ctnnb1*^{lox} allele (Brault et al., 2001), producing pancreas-specific β-catenin knockout (PBKO) mice (Fig. 1A). Confirming published results, we found that mutant pancreata were drastically smaller at late fetal stages, due mostly to the loss of exocrine acinar cells (Fig. 1B and C). To more accurately assess the effect of deleting β-catenin, we developed a strategy of absolute volume quantification, in which evenly-spaced sections were collected across a series of slides, to capture the entire pancreas (see Materials and methods). Integrating the area occupied by immunostaining for individual markers allowed us to estimate the absolute volume of pancreas occupied by specific cell types. Staining for the pan-epithelial marker E-cadherin at E17.5 revealed a 10-fold reduction in overall pancreas size in PBKO compared to controls (Fig. 1D–F). The drastic reduction in size is mostly due to the loss of exocrine acinar cells as shown previously (Murtaugh et al., 2005; Wells et al., 2007), together with a decrease in duct cells (Fig. 1J). Casual observation suggested a possible increase in β-cell numbers in PBKO, as β-cells occupied more of the remnant epithelium than in control pancreata. However, quantification revealed a 2-fold reduction in β-cell volume following β-catenin deletion, not documented in any previous study (Fig. 1G–I). The volume of other endocrine cell types, including α-cells and δ-cells, exhibited a similar reduction, representing a deficit in overall islet cell mass (Fig. 1J). Importantly, the size of β-cells was indistinguishable between wild-type and mutant (Fig. S1A–C), confirming that the decrease in β-cell volume reflected a decrease in β-cell numbers.

Because *Pdx1-Cre*^{early} deletes β-catenin in early pancreatic progenitor cells, analysis of late fetal PBKO pancreata cannot distinguish between defects that arise early, in time or lineage progression, from those arising late. We were particularly interested in establishing if the decreased β-cell mass in PBKO reflected a requirement for β-catenin in insulin+ cells themselves and/or in Ngn3+ endocrine precursors. We used an *Ngn3-Cre* BAC transgenic mouse line (Schonhoff et al., 2004) to delete *Ctnnb1* specifically in Ngn3+ precursors and their progeny, avoiding any potential effect in Pdx1+ MPCs. In contrast to *Pdx1-Cre*^{early}-mediated deletion, we found no change to β-cell mass following deletion with *Ngn3-Cre* (Fig. 1K–M). These data suggest that β-catenin acts at a stage prior to endocrine specification to establish both normal pancreas size and normal β-cell mass.

To determine the specific period in which β-catenin was required during development, we compare wild-type and mutant pancreas size at several developmental time points. Although pancreas size was not significantly affected at E11.5, β-catenin deficient pancreata were significantly smaller than control beginning at E12.5, the discrepancy increasing with time (Fig. 2). Interestingly, the timing of the PBKO pancreas hypoplasia phenotype corresponds approximately to the classically-described "secondary transition" (Pan and Wright, 2011; Pictet and Rutter, 1972), defined by an exponential increase in the numbers of differentiated

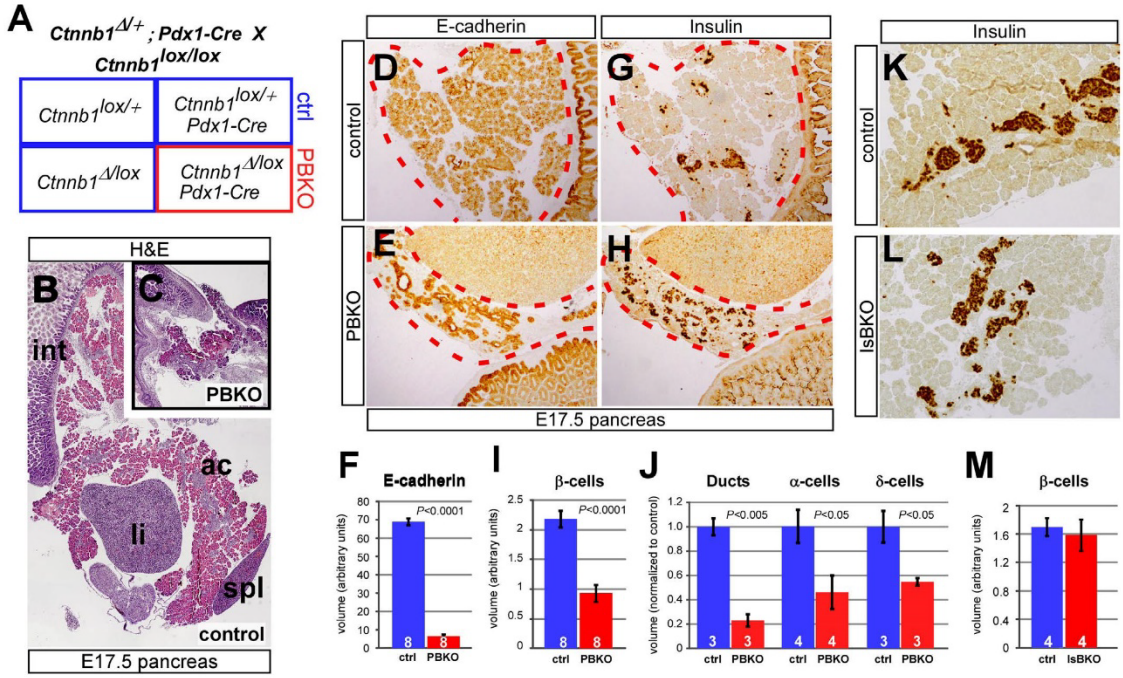

Fig. 1. β-Catenin is required for pancreas and β-cell mass prior to endocrine specification. *Pdx1-Cre* (PBKO) or *Ngn3*-Cre (IsBKO) β-catenin knockout pancreata were analyzed and compared to controls at E17.5. (A) Breeding scheme to generate control and KO genotypes. (B and C) H&E staining of E17.5 pancreata reveals a dramatic loss of acinar tissue (pink) in PBKO compared to control. (D–F) Pancreata (red-dashed outline) stained with the pan-epithelial marker E-cadherin to determine total pancreas size exhibit a 10-fold reduction in PBKOs compared to controls. (G–I) Although insulin+ β-cells occupy a greater proportion of PBKO pancreatic epithelia (adjacent sections to D and E), quantification reveals a 2-fold reduction in absolute β-cell volume. (J) Quantification of ducts (DBA-lectin+), α-cells (glucagon+), and δ-cells (somatostatin+), normalized to controls at E17.5, demonstrates that PBKOs have reduced exocrine and endocrine numbers. (K and M) IsBKO (*Ngn3-Cre)* and control pancreata show no difference in β-cell volume at E17.5. The number of embryos analyzed per genotype is indicated at the bottom of each bar.

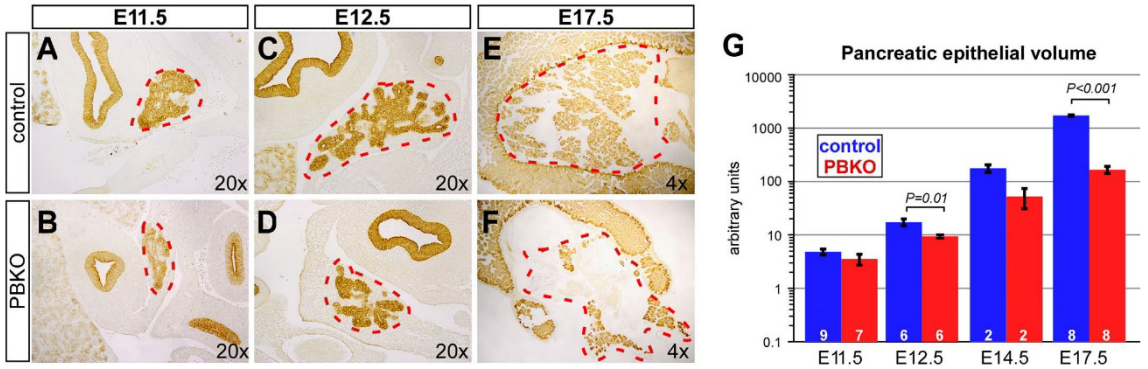

Fig. 2. Progressive growth defect in β-catenin mutant pancreata. PBKO or control pancreata from E11.5, E12.5 and E17.5 were stained with the pan-epithelial marker E-cadherin to determine overall pancreas size (dashed outline). (A and B) At E11.5, pancreas size is similar between PBKOs and controls. (C and D) At E12.5, PBKO pancreata are smaller and contain fewer distal branches than controls. (E and F) Staining for the pan-epithelial marker E-cadherin at E17.5 indicates a dramatic deficiency of epithelial tissue in PBKOs compared to controls. Control and mutant images photographed at identical magnifications, as indicated. (G) Volume quantification of E-cadherin-stained surface area (graphed on the log axis) reveals a size deficit in PBKO pancreata beginning at E12.5, increasing through E17.5.

endocrine and exocrine cells. As our quantitative analysis identified E12.5 as the first stage at which phenotypic changes could be observed in PBKO, we focused our studies on this period to characterize and quantify changes to the MPC population.

Early loss of distal MPCs in β-catenin-deficient pancreata

Lineage tracing studies reveal that all mature pancreatic cell types arise from MPCs (Zhou et al., 2007). Following specification and prior to the secondary transition, the pancreatic epithelium segregates into a MPC-containing distal tip domain and a proximal trunk domain that comprises duct and endocrine precursors (Pan and Wright, 2011; Zhou et al., 2007). To characterize any changes to the distal MPC population, we stained and quantified potential MPCs at E12.5, labeled independently with three markers, Ptf1a, carboxypeptidase 1 (Cpa1) and c-Myc (Zhou et al., 2007). We found MPC numbers to be dramatically reduced in PBKO, regardless of the marker used (Fig. 3A–C, D–F, and G–I). This reduction is not simply explained by the overall decrease in PBKO epithelial volume, as the proportion of Ptf1a+ cells within the pancreas was also dramatically reduced (Fig. S2A). The remaining Ptf1a+ cells found in PBKO pancreata likely represent "escaper" cells that fail to delete β-catenin, and later provide the only source of residual acinar cells in these embryos (Murtaugh et al., 2005). Overall, the morphology of the PBKO epithelium resembled a proximal trunk domain devoid of branching tips.

In addition to staining for distal tip MPCs, we looked for any potential defects within the proximal and pre-endocrine populations by staining for Sox9 or Ngn3, respectively. At E12.5, Sox9 is still expressed in both the distal and proximal domain, and we observed no change in the Sox9 expression pattern (Fig. S3). However, in contrast to the loss of distal MPCs, we found an unexpected increase of Ngn3+ cells, both in absolute number and as a proportion of the trunk epithelium (Figs. 3J–L and S2B). Furthermore, some Ngn3+ cells remained in the distal domain one day later, at E13.5 (data not shown). Consistent with the bias of early Ngn3+ cells toward the α-cell lineage (Johansson et al., 2007), we found a modest increase in glucagon+ cells in PBKOs at this time, suggesting that extra Ngn3+ cells present at E12.5 in PBKOs had differentiated (Fig. S4). Given that acinar cells arise from distal tip MPCs persisting at the secondary transition (Zhou et al., 2007), our data suggest that the acinar deficit of PBKO mice reflects an earlier defect in maintenance of distal tip identity, such that β-catenin-deficient distal MPCs take on proximal "trunk" cell characteristics.

Pancreatic patterning is established but not maintained in PBKO

Given that β-catenin-deficient pancreata contained fewer distal MPCs at E12.5 than controls, we sought to determine whether β-catenin was required for establishing or maintaining MPC identity and distal patterning. We identified MPCs at E11.5, a time point prior to any noticeable decrease in total epithelial size, based on their expression of Ptf1a or c-Myc (Zhou et al., 2007), and found no significant difference in the number of Ptf1a+ cells between controls and PBKOs (Fig. 4A–C). The number of c-Myc+ cells, however, was already reduced in E11.5 PBKOs compared to controls (Fig. 4D–F), similar to our findings at E12.5. Importantly, c-Myc is a β-catenin target gene (He et al., 1998) and known to promote pancreatic epithelial proliferation (Bonal et al., 2009; Nakhai et al., 2008), suggesting that its loss of expression reflected loss of β-catenin signaling activity.

Interestingly, the total number of Ptf1a+ cells present in E11.5 PBKOs was greater than the number at E12.5 (Fig. 4C), suggesting that the deficit observed at the later stage may reflect more than a simple failure to expand. Confirming previous results (Murtaugh et al., 2005), we found no detectable increase in cell death in PBKO pancreata at these stages (data not shown). We therefore interpret our results as indicating normal MPC specification at E11.5, followed by a progressive shift from distal to proximal fate of these cells in the absence of β-catenin function.

To test the hypothesis that β-catenin acts post-specification to support MPC maintenance and acinar development, we used the tamoxifen-inducible *Pdx1-CreERT* deletor (Gu et al., 2002) to achieve temporal control of *Ctnnb1* recombination. We also bred in the Cre-dependent *R26R*^{EYFP} reporter (Srinivas et al., 2001), allowing for lineage tracing of EYFP+/β-catenin-deficient cells. These mutant embryos are referred to as mPBKO, for mosaic pancreas-specific β-catenin knockout. We first administered tamoxifen to pregnant dams at E10.5 and harvested pancreata at E17.5, analyzing EYFP distribution to determine the fate of manipulated cells. Our previous study using constitutive *Pdx1-Cre* indicated that β-catenin-deficient cells are excluded from the acinar lineage while remaining competent for β-cell differentiation (Murtaugh et al., 2005). Consistent with this, we found a dramatic reduction in the acinar contribution of EYFP+ cells in mPBKO pancreata following E10.5 TM treatment, while β-cell

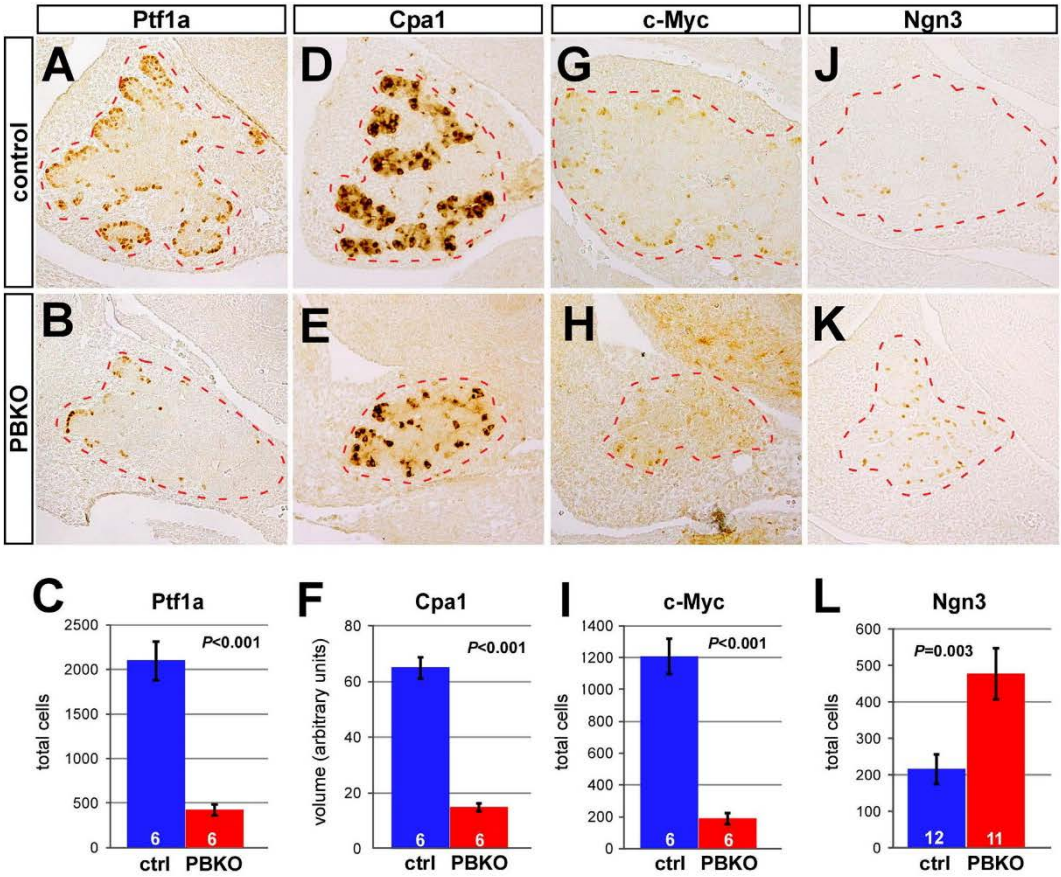

Fig. 3. Proximalization of β-catenin deficient pancreata during MPC expansion. E12.5 mutant and control pancreata were stained for Ptf1a, Cpa1 and c-Myc to label distal MPCs, and Ngn3 to label endocrine precursors (epithelia are outlined in red). (A–C) Control Ptf1a staining identifies MPCs localized to the distal tips of the expanding epithelium. PBKO pancreata contained fewer distal tips and fewer Ptf1a+ cells than age-matched controls, confirmed by quantification. (D–F) Similar to Ptf1a, the distal MPC marker Cpa1 exhibits a dramatic decrease in PBKOs compared to controls. (G–I) Cells expressing the Wnt target gene c-Myc are identified in distal tips of control but not PBKO pancreata. (J–L) Staining for the endocrine precursor marker Ngn3shows an absolute increase in Ngn3+ cells in PBKOs, with some Ngn3+ cells localizing abnormally to distal tips rather than being confined to the proximal trunk domain. Quantification of Ptf1a, c-Myc and Ngn3 is represented as the total number of cells calculated per pancreas.

labeling remained comparable to controls (Fig. S5). Our mosaic deletion/labeling approach thus provides independent evidence that β-catenin is required in the acinar lineage, and indicates that this requirement applies during stages of MPC expansion.

To examine more closely the fate of β-catenin deficient MPCs, we administered tamoxifen at either E8.5 (during/prior to MPC specification) or E10.5 (following specification), and harvested embryos 3 days later at either E11.5 or E13.5, respectively. In the E8.5–E11.5 pulse-chase paradigm, we found that Cpa1+ MPCs exhibited a similar EYFP labeling index in control and mPBKO (Fig. 5A–C), consistent with a lack of requirement for β-catenin in the initial generation of MPCs. Staining for β-catenin protein confirmed that labeled cells in mPBKO pancreata were β-catenin-deficient (Fig. 5B). In the E10.5–E13.5 paradigm, however, we found that Cpa1+ cells were labeled much less frequently in mPBKOs than in controls, and EYFP+ cells were generally excluded from distal tips and confined to proximal trunk regions (Fig. 5D–F). Moreover, we found that the majority of EYFP+ Cpa1+ cells in E10.5–E13.5 mPBKOs were "escapers" that had not successfully deleted *Ctnnb1*, as indicated by β-catenin staining (Fig. 5E). Together with the results of our PBKO studies, above, these lineage tracing experiments suggest that β-catenin is required for MPC maintenance and proximal–distal patterning, and acts during MPC expansion rather than specification.

β-Catenin regulates proliferation independent of proximal–distal patterning

Intuitively, if MPC numbers are determined by integrating their self-renewal with differentiation to a bipotent proximal fate (Zhou et al., 2007), then the progressive MPC depletion we observe in PBKO between E11.5 and E12.5 (Fig. 4C) could reflect a growth arrest of these cells. Indeed, Wnt/β-catenin signaling is well known to regulate proliferation in many tissues, including mature acinar cells (Keefe et al., 2012; Polakis, 2012; Schuijers and Clevers, 2012), and decreased proliferation has been documented in β-catenin mutant pancreata, albeit at isolated time points and without reference to specific cell types affected (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007).

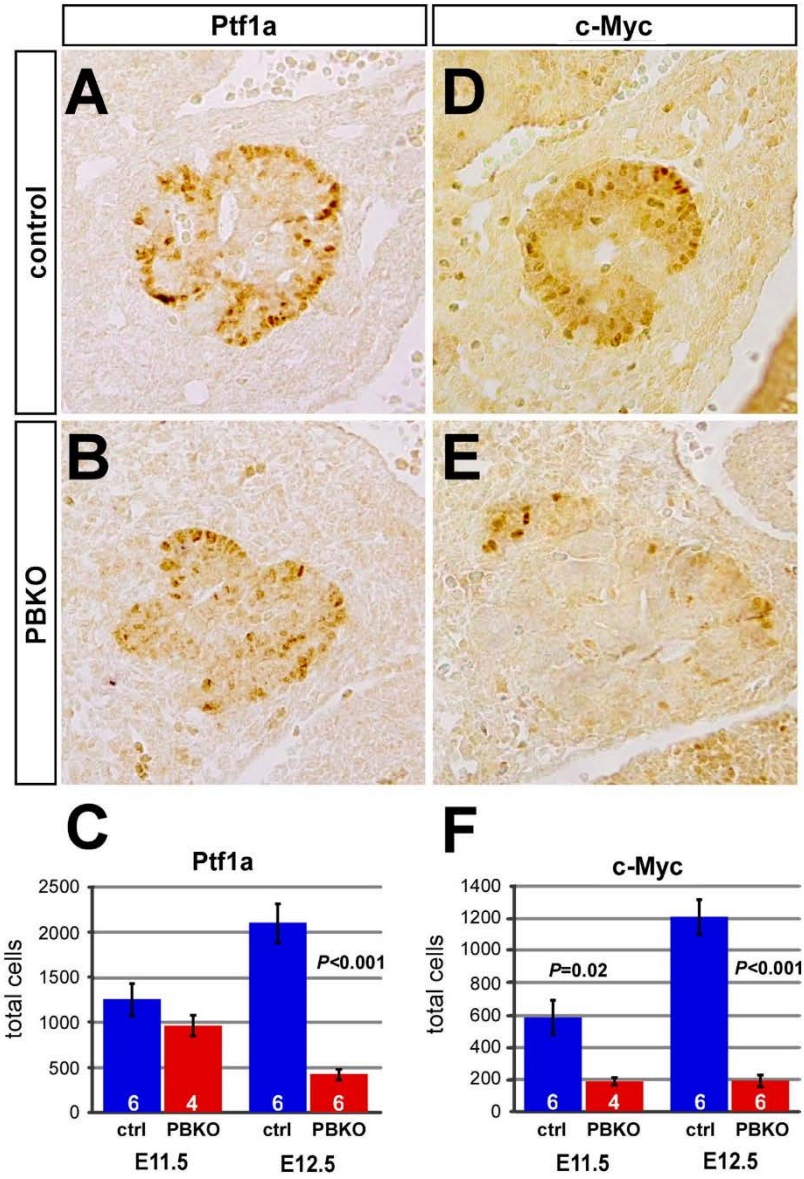

Fig. 4. Proximal–distal patterning is established in the absence of β-catenin. E11.5 pancreata were stained with Ptf1a and c-Myc to label and quantify distal MPCs. Total cell number indicates the number of labeled cells calculated per pancreas. (A and B) Ptf1a staining is similar between control and PBKO at this stage, with Ptf1a+ cells localized to the distal periphery. (C) Ptf1a+ cells at E11.5 show a non-significant difference between PBKO and control. The total number of Ptf1a+ cells (reproduced from Fig. 3C) increased in controls from E11.5 to E12.5, and decreased in PBKOs. (D and E) Staining for c-Myc reveals a decrease in the number of c-Myc+ cells in PBKOs compared to controls. (F) C-Myc+ cells are markedly decreased in E11.5 PBKO pancreata.

To determine the spatiotemporal requirements for β-catenin in MPC proliferation, we injected pregnant mice with BrdU to label proliferating cells one hour prior to harvest at E11.5, E12.5 and E13.5, and analyzed BrdU labeling of Cpa1+ distal MPCs. Surprisingly, we found similar levels of proliferation between controls and PBKOs at E11.5 (Fig. 6A–F). Beginning at E12.5 and continuing through E13.5, however, PBKO cells incorporated BrdU less frequently than age-matched controls (Fig. 6G–L and M–R). We calculated the percentage of BrdU+ cells among all pancreatic epithelial cells, marked in this experiment by activation of *R26R*^{EYFP}, as well as in Cpa1+ cells specifically, and found no significant difference in proliferation in either population at E11.5. Cpa1+ cell proliferation remained constant in controls through all periods, whereas overall proliferation begins to decline at E13.5. In PBKOs, both overall and Cpa1+ cell proliferation fell dramatically at E12.5 and remained reduced at E13.5 (Fig. 6S and T). With little or no proliferation defect in mutant MPCs at E11.5, their rapid disappearance over the next 24 h is most readily explained by a

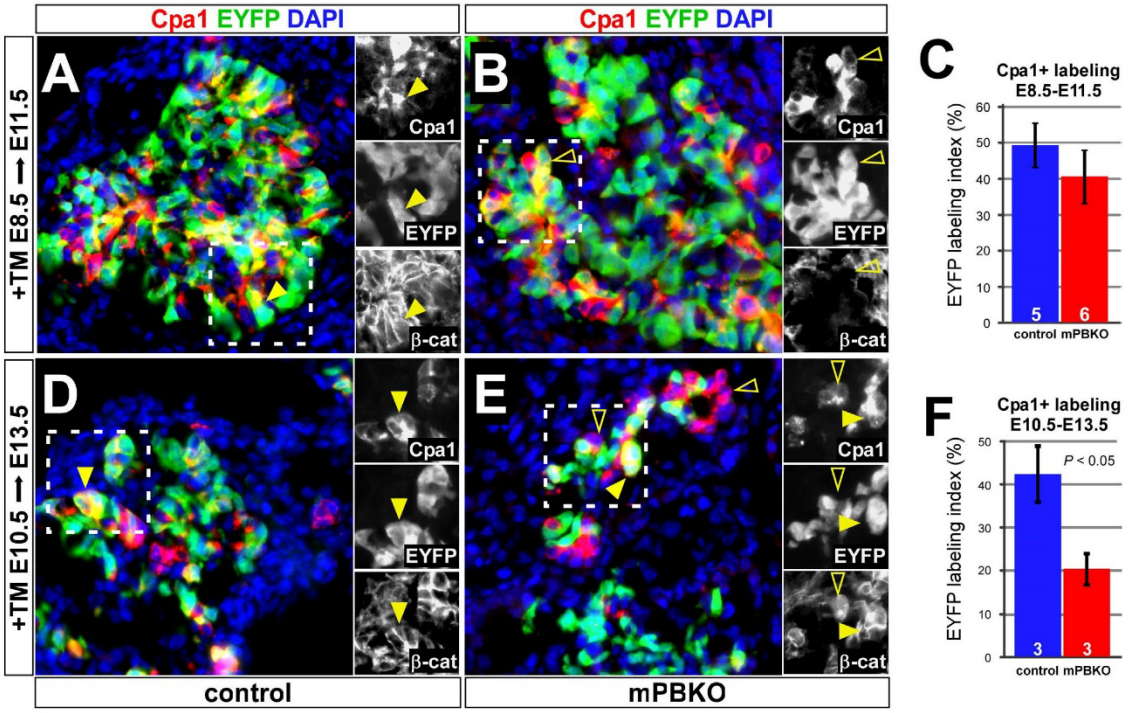

Fig. 5. β-Catenin is required to maintain but not establish distal tip cell identity. Control (*Ctnnb1*$^{lox/+}$; *R26R*$^{EYFP/+}$; and *Pdx1-CreERT/+*) and mosaic pancreas-specific β-catenin knockout (mPBKO: *Ctnnb1*$^{\Delta/lox}$; *R26R*$^{EYFP/+}$; and *Pdx1-CreERT/+*) embryos received tamoxifen in utero at either E8.5 or E10.5 and were analyzed 3 days later (E11.5 and E13.5, respectively) by immunofluorescence. (A) Cpa1+ distal cells (red) from control pancreata given tamoxifen at E8.5 and harvested at E11.5 co-express EYFP (green) as indicated by yellow arrowheads. Monochrome panels depict single color channels for Cpa1 and EYFP, from area highlighted in dashed box, together with β-catenin protein (not included in the merge, but taken from the same field), and confirm that β-catenin is expressed by EYFP+ Cpa1+ cells in control (closed arrowheads). (B) Cpa1+ cells (red) from mPBKO littermates co-express EYFP (green). Single color channels indicate that most EYFP-labeled Cpa1+ cells are β-catenin-deficient (open arrowheads). (C) The EYFP labeling indices of Cpa1+ cells remain similar between controls and mPBKO. (D) Cpa1+ distal tip cells from controls given tamoxifen at E10.5 and harvested at E13.5 co-express EYFP and β-catenin. (E) In mPBKO littermates, EYFP+ and β-catenin-deficient cells are generally excluded from distal Cpa1+ tips. Remaining Cpa1+ EYFP+ cells retain β-catenin expression, indicating a selection for MPCs that have not deleted β-catenin (closed arrowheads). Open arrowheads indicate unlabeled, β-catenin+ Cpa1+ cells that comprise a large majority of the Cpa1+ population. (F) After an E10.5 TM pulse, control Cpa1+ cells retain a higher EYFP labeling index than mPBKO.

key requirement for β-catenin in distalization of the epithelium. Our results suggest that the loss of MPCs, through altered patterning leading to premature differentiation as well as through reduced proliferation, ultimately diminishes influx into the pool of precursors that generates β-cells later in development (Fig. 7H).

Notch signaling is required for proximalization induced by loss of β-catenin

Elimination of β-catenin and elimination of Notch signaling have opposite patterning consequences in the developing pancreas: whereas loss of β-catenin produces a shift toward proximal/trunk patterning, the inhibition of Notch promotes distal/tip cell development (Afelik et al., 2012; Magenheim et al., 2011). Moreover, ectopic activation of Notch prevents acinar cell differentiation, similar to β-catenin loss-of-function (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003). Notch and Wnt/β-catenin have been found to have antagonistic effects in other experimental systems as well, although this appears to be context-specific (Acosta et al., 2011; Hayward et al., 2005; Kwon et al., 2011). To determine the relationship between these pathways in the pancreas, we cultured E11.5 PBKO pancreata in the absence or presence of the gamma-secretase inhibitor DBZ, which inhibits ligand-induced Notch receptor activation (Magenheim et al., 2011; Milano et al., 2004). After 3 days of culture, untreated PBKO explants developed few Cpa1+ tip/pro-acinar cells, most of which represented β-catenin+ "escaper" cells (Fig. 7A–C). DBZ-treated PBKO explants, however, developed large numbers of Cpa1+ cells within β-catenin deficient areas (Fig. 7D–F). Notably, the number of Cpa1+ escaper cells was not increased by DBZ, indicating that Notch inhibition did not induce generalize hyperplasia of tip/pro-acinar cells (Fig. 7G). These results provide insight into the mechanism by which β-catenin normally promotes distal fate, namely by inhibiting the ability of Notch to drive tip-to-trunk conversion (Fig. 7H).

Discussion

We previously found that the multifunctional protein β-catenin was required for pancreatic acinar development but dispensable for endocrine differentiation and function (Murtaugh et al., 2005). We have revisited the pancreas-specific β-catenin knockout model to reveal a requirement for β-catenin in generating normal islet cell numbers and, through the use of lineage-restricted Cre deletor lines, we demonstrate that this requirement is imposed prior to endocrine specification. β-catenin has two distinct roles in the early pancreas: it promotes distal patterning of the epithelium by inhibiting Notch, and it is required for maximal proliferation throughout the developing organ (Fig. 7). Our findings provide

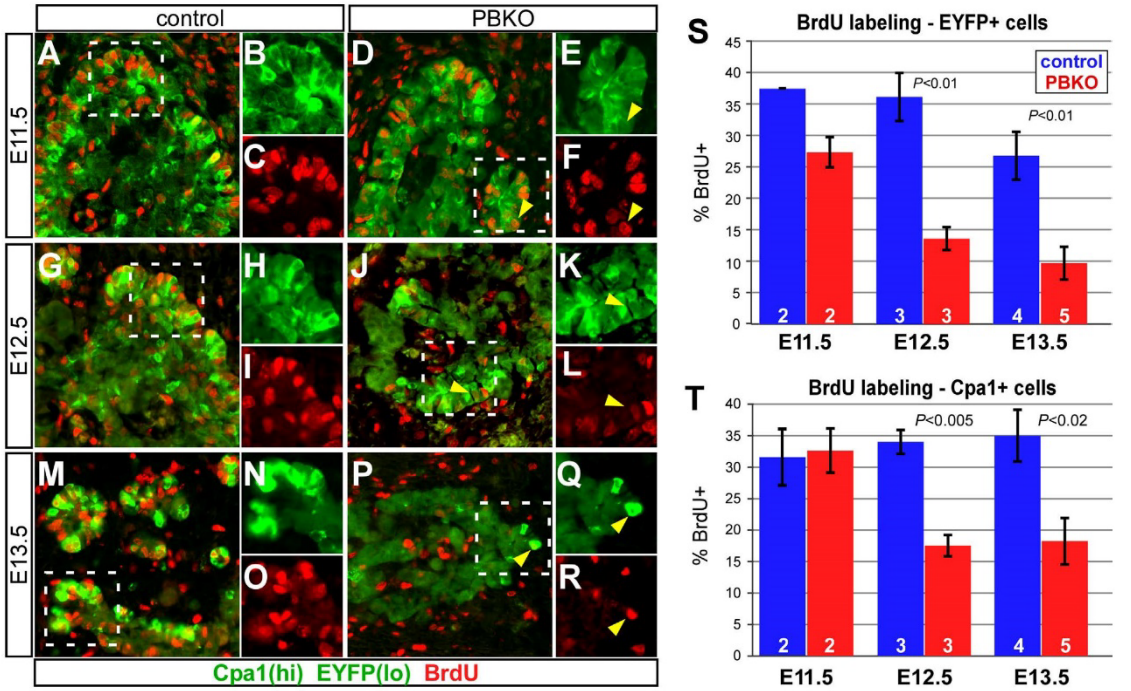

Fig. 6. β-Catenin is required after E11.5 for epithelial proliferation. Control (*Ctnnb1*$^{lox/+}$; *R26R*$^{EYFP/+}$; and *Pdx1-Cre*/+) and PBKO (*Ctnnb1*$^{\Delta/lox}$; *R26R*$^{EYFP/+}$; and *Pdx1-Cre*/+) littermates received BrdU in utero one hour prior to sacrifice at E11.5, E12.5 or E13.5, and proliferation index was assessed via BrdU immunostaining (red). All pancreatic epithelial cells are EYFP-labeled (light green) due to *Pdx1-Cre* recombination of *R26R*^{EYFP}, while Cpa1+ cells appear as bright green. White boxes indicate areas with expanded views seen in the adjacent panels. (A–C) E11.5 controls contain both BrdU+ Cpa1+ distal and BrdU+ Cpa1 -proximal cells. (D–F) Distal cells from PBKO at E11.5 are BrdU labeled at levels comparable to controls (yellow arrowheads). (G–I) At E12.5, comparable BrdU labeling is seen between Cpa1+ and Cpa1- of controls. (J–L) BrdU labeling is reduced in E12.5 PBKOs, regardless of Cpa1 expression status. (M–O) Distal cells are commonly BrdU labeled in E13.5 controls. (P–R) BrdU+ cells are dramatically reduced throughout the PBKO epithelium at E13. (S) BrdU labeling indices for EYFP+ cells at E11.5, E12.5 and E13.5 reveal that maximal epithelial proliferation requires β-catenin function only after E11.5. (T) Proliferation indices calculated for the Cpa1+ population in PBKOs and controls.

new insight into the molecular mechanisms of pancreas morphogenesis and differentiation, as well as reconcile previous studies of β-catenin knockout pancreata.

Whereas several prior studies found no change in islet cell number or function following deletion of β-catenin (Murtaugh et al., 2005; Wells et al., 2007), a separate study suggested that islet cell numbers declined following β-catenin deletion, reflecting islet-restricted Wnt-β-catenin transcriptional activity (Dessimoz et al., 2005). To avoid problems of overestimating β-cell mass due to changes in the overall organ structure (Kopp et al., 2011), we used a quantitative approach that captures the absolute rather than relative volume occupied by specific pancreatic cell types, and discovered a more drastic loss of β-cells in mutant pancreata than previously reported. However, and consistent with our recent finding that postnatal β-cell proliferation is β-catenin-independent (Keefe et al., 2012), we find that islet precursor-restricted deletion of β-catenin does not recapitulate the loss of islet mass caused by deletion in MPCs. Therefore, we conclude that β-catenin acts prior to endocrine specification, maintaining and expanding MPC numbers sufficient to establish normal islet mass (Stanger et al., 2007). In the absence of β-catenin, MPCs are proximalized and prematurely generate early endocrine precursors, biased toward glucagon+ α-cells (Johansson et al., 2007), and are not subsequently available in sufficient numbers to generate the full complement of β-cells. Overall decreased islet cell genesis in the absence of β-catenin may also reflect antagonism between this protein and Notch, uncovered here, as hyperactive Notch is known to inhibit endocrine development (Hald et al., 2003; Murtaugh et al., 2003). Further studies will be required to parse the roles of β-catenin and Notch in MPCs and trunk cells.

How does β-catenin regulate MPC numbers? Importantly, we find that the initial specification, patterning and proliferation of MPCs are normal in PBKO pancreata, through E11.5, but notably aberrant as soon as 24 h later. In particular, the progressive disappearance of MPCs, and the increased number of Ngn3+ cells, indicates a defect in maintaining the early proximal–distal pattern of the organ, independent of effects on proliferation. Proliferation is impaired in PBKO pancreata, a defect that may reflect, in part, loss of expression of the Wnt target gene *cMyc* (Bonal et al., 2009; Nakhai et al., 2008). Direct inhibition of Wnt signaling in the pancreas, through expression of a dominant-negative *Fz8* mutant, impairs proliferation without any obvious effect on patterning or differentiation (Papadopoulou and Edlund, 2005). We are therefore tempted to speculate that the proliferation-specific effects of β-catenin reflect activity of the canonical Wnt signaling pathway, while effects on patterning may reflect Wnt-independent β-catenin functions, possibly including cross-talk with Notch. In future studies, we hope to address this issue with new genetic tools that we and others have developed to selectively disrupt Wnt/β-catenin signaling (Barrott et al., 2011; Valenta et al., 2011). If β-catenin has distinct modes of action, it may be possible to selectively proximalize early epithelial cells, promoting islet precursor development, while maintaining normal or even increased proliferation of progenitor cells. Such an intervention could be critical to efficiently generate β-cells from human ES or iPS cells.

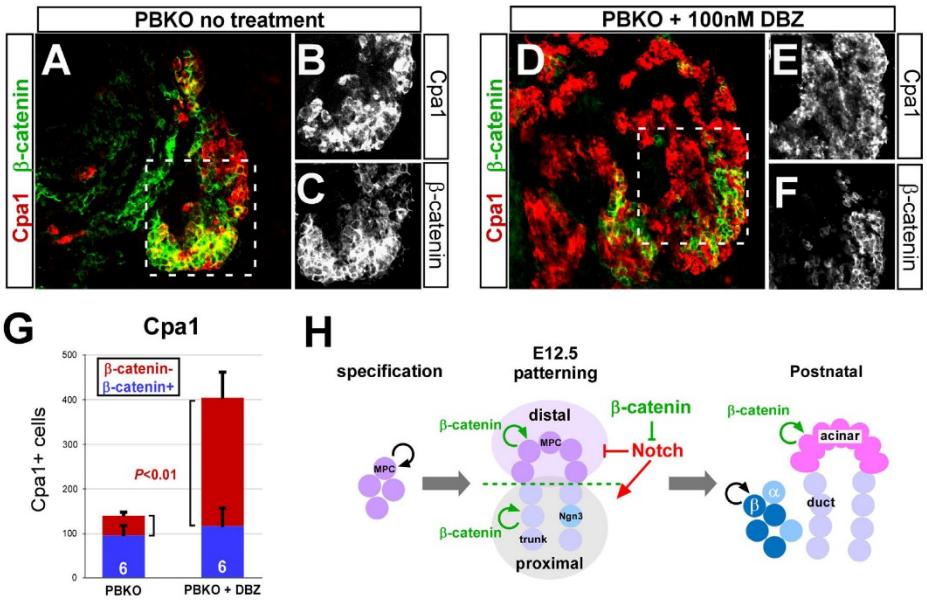

Fig. 7. Notch inhibition rescues tip/pro-acinar specification of β-catenin-deficient pancreata. PBKO and littermate control explants were cultured for 3 days with or without the γ-secretase-inhibitor DBZ (100 nM). Explants were stained by wholemount immunofluorescence with Cpa1 (red) and β-catenin (green). (A–C) Most Cpa1+ cells present in untreated PBKO explants are also β-catenin+. (D–F) The majority of Cpa1+ cells are β-catenin-deficient in DBZ treated PBKO explants. (G) Quantification of the total number of Cpa1+ cells, scored as β-catenin+ (blue portion of bar) or β-catenin- (red portion of bar), shows a significant increase in the number of Cpa1+ β-catenin- cells in DBZ treated PBKO explants. (H) We have identified a role for β-catenin in maintaining the distal pattern of early pancreatic progenitor cells, indicated by the green dashed line between distal and proximal domains at E12.5. This function appears to be mediated in part by inhibition of Notch, which induces distal-to-proximal differentiation of MPCs. Independent of its role in patterning, β-catenin is also required for maximal proliferation of tip and trunk progenitors as well as acinar cells (green circular arrows), but not for newly specified MPCs or postnatal β-cells (black circular arrows) (Keefe et al., 2012).

The fact that β-catenin is essentially dispensable before E12.5 may explain the previous paradoxical finding that early activation of β-catenin actually causes pancreas agenesis (Heiser et al., 2006). This result was obtained with *Pdx1-Cre*^{early}, which induces recombination prior to MPC specification; activation of β-catenin following the secondary transition, with *Pdx1-Cre*^{late}, results in exocrine pancreas hyperplasia. We suggest that the signaling function of β-catenin is normally inactive prior to MPC specification, and that its hyperactivation at early stages may respecify the organ to an alternative developmental fate (Heller et al., 2002; McLin et al., 2007).

The basis for the transition to β-catenin dependence, for both patterning and proliferation, remains unknown. We note, however, that it appears to occur simultaneously with several other regulatory transitions, including resolution of epithelial branching (Villasenor et al., 2010), establishment of mutually repressive Nkx6.1 and Ptf1a expression domains (Schaffer et al., 2010), and upregulation of the *Ptf1a* autoregulatory enhancer (Masui et al., 2008). Our results would be consistent with any or all of these transitions requiring β-catenin function, and we are interested to determine the mechanism or mechanisms by which β-catenin orchestrates these and other early processes to allow subsequent elaboration of organ growth and differentiation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.03.019.

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SUPPLEMENTAL MATERIAL

Table S1: Primary antibodies used in this study

Antigen	Species	Source	Catalog #	Dilution
Amylase	Rabbit	Sigma	A8273	1:1000
BrdU	Rat	Abcam	AB6326	1:2000
c-Myc	Rabbit	Cell Signaling	5605	1:500
C-peptide	Rabbit	Linco	4020-01	1:2500
Cpa1	Goat	R&D Systems	AF2765	1:1000
E-cadherin	Goat	R&D Systems	AF748	1:2000
GFP	Goat	Rockland	600-101-215	1:5000
GFP	Rabbit	Abcam	AB290	1:4000
Glucagon	Guinea pig	Linco	4031-01F	1:2500
Insulin	Guinea pig	Dako	A-0564	1.2000
Ngn3	Mouse	Developmental Studies Hybridoma Bank	I25A1B3	1:75
Ptf1a	Rabbit	Chris Wright, Vanderbilt University		1:5000
Somatostatin	Goat	Santa Cruz Biotechnology	SC-7819	1:500
Sox9	Rabbit	Millipore	AB5535	1:1000

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1: β-cell size is unchanged in the absence of β-catenin. (A-C) β-cell size at E17.5 was calculated by dividing the area of insulin staining (red) by the number of overlapping DAPI+ cells (blue). **(A-B)** E17.5 β-cells in controls and PBKO appear similar in size. **(C)** No significant β-cell size difference was found between control and PBKO at E17.5.

Supplementary Figure 2: β-catenin maintains epithelial proportions of distal tips and Ngn3+ cells. E12.5 pancreata were stained with E-cadherin to label all cells, Ptf1a to label distal tips and Ngn3 to label endocrine precursors. **(A)** The percentage of all pancreatic E-cadherin+ cells that were also Ptf1a+ decreases in PBKOs compared to controls. **(B)** The percentage of Ngn3+ cells found within the trunk epithelium (Pft1a-, E-cadherin+) was increased in PBKOs compared to controls, suggesting a shift toward proximal trunk/endocrine fate in the absence of β-catenin.

Supplementary Figure 3: Sox9 expression is unchanged in the absence of β-catenin. E12.5 pancreata were stained with Sox9 and E-cadherin. **(A)** Controls show nearly ubiquitous staining of the pancreatic epithelium (green) with Sox9 (red) at E12.5, with staining absent from likely αcells (arrowhead). **(B)** Sox9 staining shown without E-cadherin in (A). **(C-D)** Staining of PBKOs appears similar to controls.

Supplementary Figure 4: E13.5 α-cell number in the absence of β-catenin. (A-B) E13.5 pancreatic epithelia were stained for glucagon (brown) to label α-cells. Control

pancreata contain centrally located α-cells, while α-cells appear to be distributed more widely in PBKO pancreata. **(C)** Quantification of total glucagon+ volume (present through the entire pancreas) indicates that PBKO pancreata trend toward a slight increase in α-cell mass (*P=0.17*).

Supplementary Figure 5: Mosaic deletion confirms that β-catenin is dispensable for β-cell differentiation. Control (*Ctnnb1$^{lox/+}$; R26R$^{EYFP/+,}$ Pdx1-CreERT/+*) and mPBKO (*Ctnnb1$^{\Delta/lox}$; R26R$^{EYFP/+,}$ Pdx1-CreERT/+*) embryos received tamoxifen in utero at E10.5, and were harvested at E17.5. **(A-C)** Staining for amylase (red) reveals dramatically decreased EYFP labeling (green) in mPBKO compared to control. **(D-F)** EYFP labeling of insulin+ β-cells (red) is similar between control and ABKO pancreata.

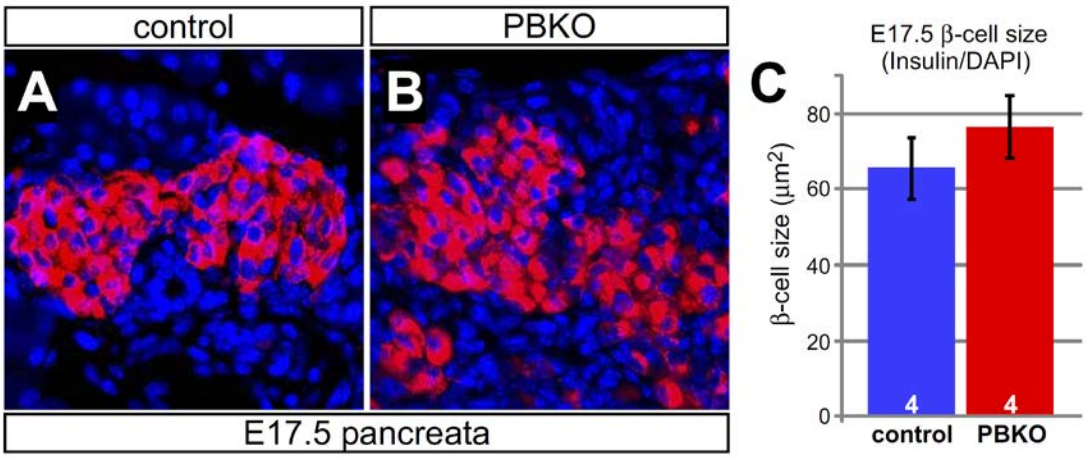
control
PBKO
A
B
E17.5 pancreata
C
E17.5 β-cell size
(Insulin/DAPI)
β-cell size (μm2)
80
60
40
20
0
4
4
control
PBKO

FIGURE S1

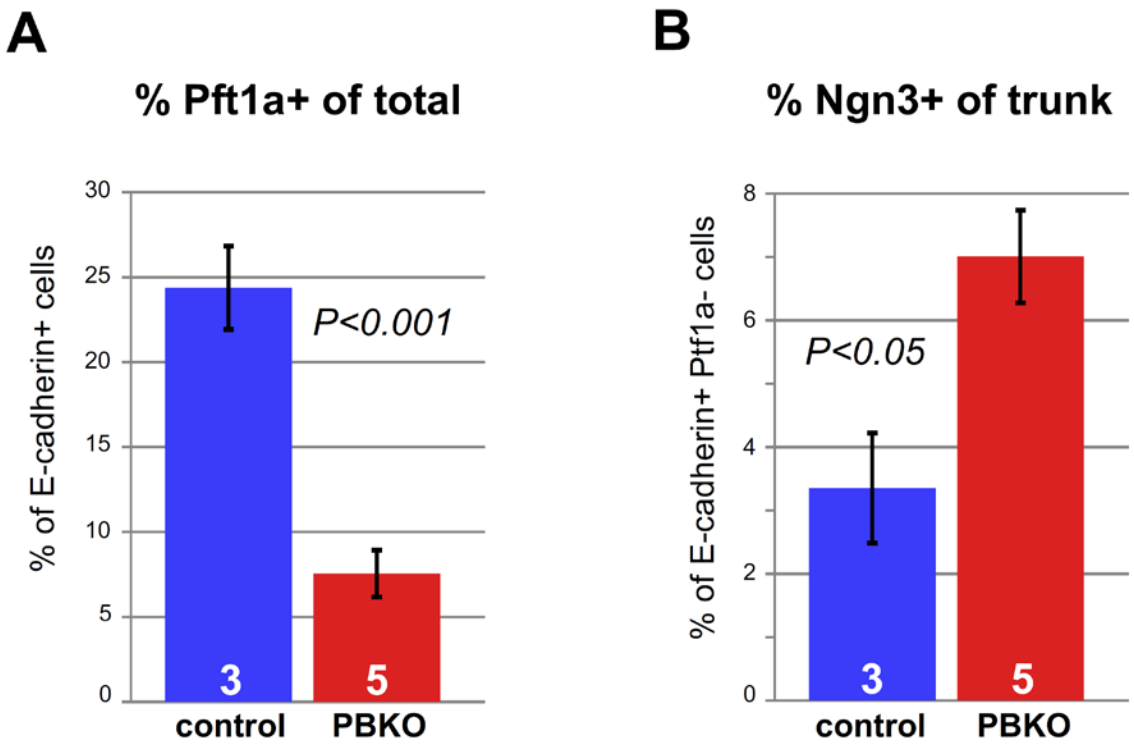
A
% Pft1a+ of total
% of E-cadherin+ cells
30
25
20
15
10
5
0
P<0.001
3
5
control
PBKO
B
% Ngn3+ of trunk
% of E-cadherin+ Ptf1a- cells
8
6
4
2
0
P<0.05
3
5
control
PBKO

FIGURE S2

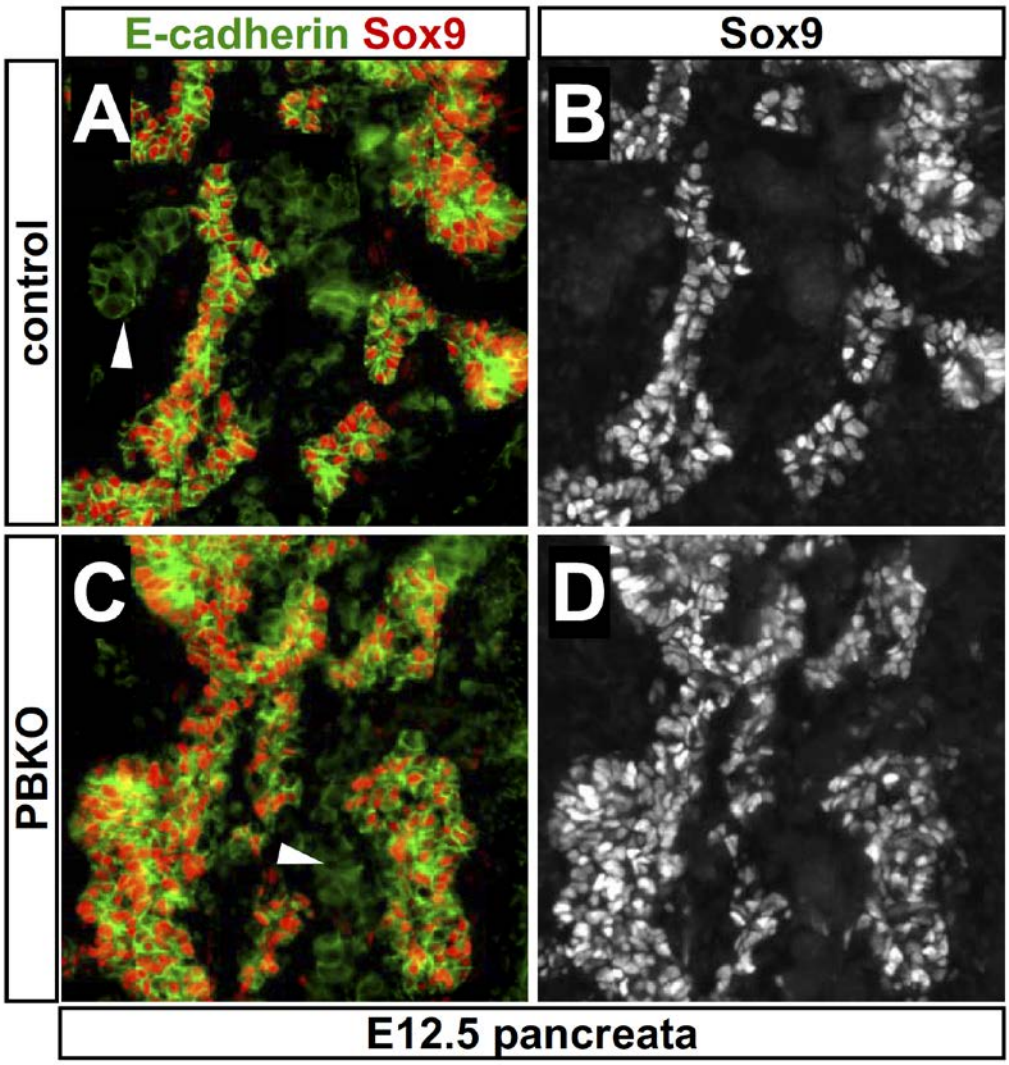

FIGURE S3

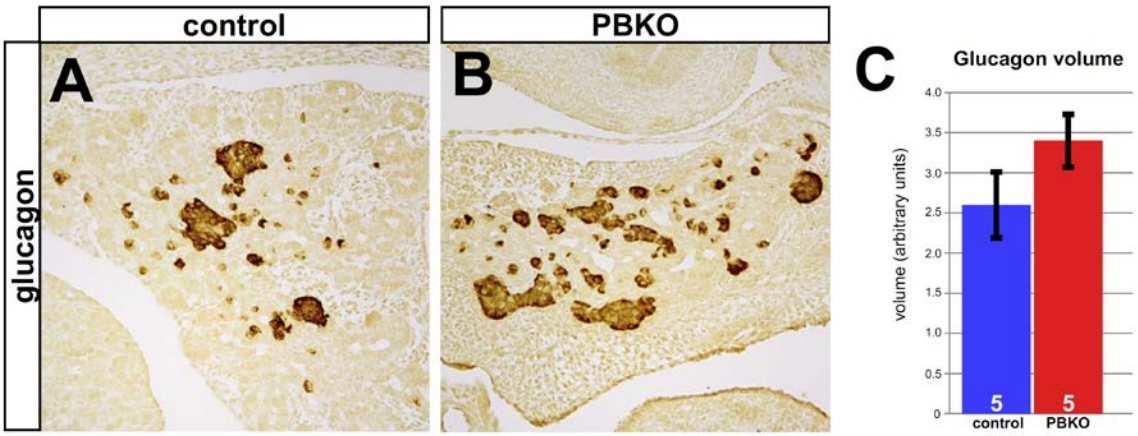
control
PBKO
glucagon
A
B
C
Glucagon volume
volume (arbitrary units)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
5
5
control
PBKO

FIGURE S4

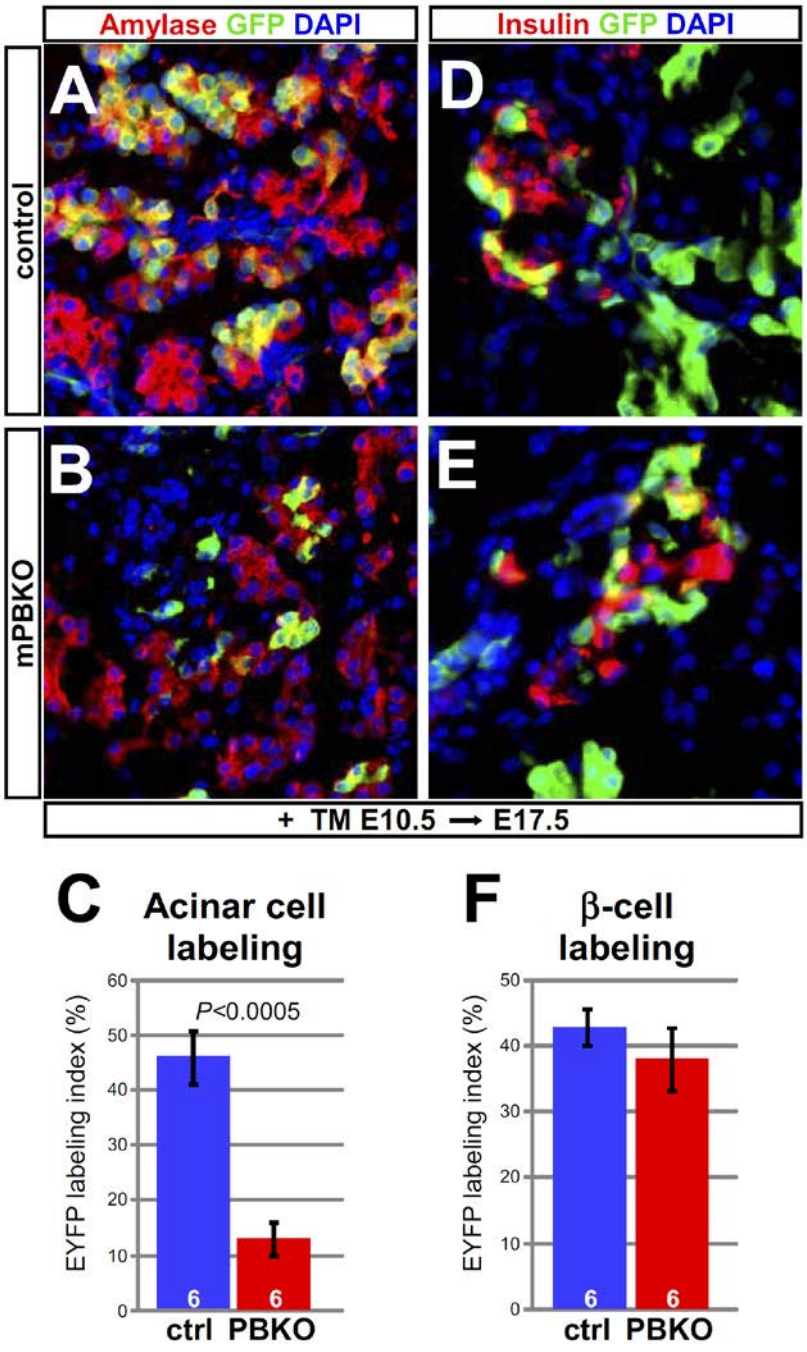
Amylase GFP DAPI
Insulin GFP DAPI
A
D
B
E
control
mPBKO
+ TM E10.5 → E17.5
C
Acinar cell labeling
P<0.0005
EYFP labeling index (%)
60
50
40
30
20
10
0
6
6
ctrl
PBKO
F
β-cell labeling
EYFP labeling index (%)
50
40
30
20
10
0
6
6
ctrl
PBKO

FIGURE S5

CHAPTER 3

DEFINING THE SIGNALING AND STRUCTURAL FUNCTIONS OF BETA-CATENIN IN PANCREAS DEVELOPMENT

Introduction

β-catenin is an evolutionarily conserved protein that regulates a vast number of processes in metazoans, from gastrulation through organogenesis during development as well as stem cell and metabolic homeostasis in adults [1]. β-catenin accomplishes such control through two distinct functional mechanisms, one structural and one signaling in nature. In epithelial cells, β-catenin functions as a component of cadherin-based cell-cell junctions, in which it binds to E-cadherin and α-catenin, thereby linking, polarizing, and stabilizing actin filament structures across neighboring cells [2]. The other central role of β-catenin is in canonical Wnt signaling, in which the presence of Wnt ligands causes accumulation of cytoplasmic β-catenin, which enters the nucleus, binds to TCF/LEF transcription factors, and recruits transcriptional co-activators to effect changes in gene expression. The majority of genetic studies of β-catenin in mice involve the tissue-specific ablation of all β-catenin function, producing a wide variety of developmental and adult phenotypes [4]. However, the majority of these studies focus on the Wnt signaling

role of β-catenin and, partly due to the lack of appropriate genetic tools, failed to address changes in cell-cell adhesion that could explain the observed phenotypes.

Careful characterization of mutations in *Drosophila* armadillo, the β-catenin homologue, revealed some mutations that affected either signaling or structural functions but not both [5], indicating that the signaling and structural functions of Armadillo/β-catenin could be separated. Using information gleaned from mapping such mutations, along with binding domain analysis, a signaling-deficient, structurally-competent allele of β-catenin was developed in the laboratory of Konrad Basler [6]. By mutating a single residue at the N-terminus as well as truncating the C-terminus of the protein, regions required to recruit transcriptional co-activators, this group created a signaling-deficient allele of β-catenin (β-catenin-double-mutant, $Ctnnb1^{DM}$), and found that the signaling and structural roles of β-catenin act separately to control the maintenance, expansion, and differentiation of neural progenitors [6]. Whether other developmental processes previously attributed to the Wnt signaling function of β-catenin are also regulated wholly or in part by its structural role is unknown. Among these is the role of β-catenin in the developing and adult pancreas, previously studied by our lab and others [7].

We have demonstrated that β-catenin is required for development, postnatal expansion, and regeneration of exocrine acinar cells, but that β-cells differentiate, function, and replicate normally in the absence of any β-catenin activity[8,9]. Despite the normal differentiation and function of β-cells, my recent work on β-catenin-deficient pancreata (Chapter 2) revealed a previously-unrecognized requirement for β-catenin in multipotent progenitor cells (MPCs), prior to endocrine specification, to generate normal islet cell numbers. Furthermore, I found that β-catenin performs two distinct functions in

the early pancreatic epithelium where it (1) maintains distal-proximal patterning following organ specification and (2) promotes proliferation driving pancreas growth [3]. However, our previous studies were performed using pancreata in which both the signaling and structural functions of β-catenin had been eliminated. Thus, which function of β-catenin controls early distal-proximal patterning, acinar differentiation, and distal-acinar cell proliferation remains unknown.

In fact, recent studies indicate a role for epithelial and cytoskeletal rearrangements in the developing pancreas, in which β-catenin might participate via its structural function [10-12]. Reorganization and stratification of the newly specified pancreatic epithelium is a necessary morphological change that establishes two specific domains, distal/tip cells and proximal/trunk cells. Tip cells, so-called for their peripheral location in the developing pancreas, contain the MPCs that produce all pancreatic lineages until the occurrence of the secondary transition at E13.5 [13,14]. Trunks cells, by contrast, function as bi-potent endocrine and duct progenitors. Thus, establishing distal-proximal division of the developing pancreas is crucial to its mature functionality. My previous work (Chapter 2) demonstrated that β-catenin is required for maintaining distal tip MPCs, supporting development of both exocrine and endocrine lineages. In the present study, we readdress the mechanistic roles of β-catenin during pancreas development and postnatal acinar cell growth, and demonstrate that its signaling and structural functions likely control different aspects of pancreatic organogenesis.

Materials and methods

Mice

All experiments were performed according to protocols approved by the University of Utah Institutional Animal Care and Use Committee. We obtained signaling-specific β-catenin mutant mice (*Ctnnb1*^{tm3Kba}, referred to as *Ctnnb1*^{DM}) from Konrad Basler (University of Zurich) [6]. Several other mouse strains were purchased from the Jackson Laboratory: floxed and germline β-catenin loss-of-function mice (*Ctnnb1*$^{tm2Kem/J}$ and *Ctnnb1*$^{tm2.1Kem}$, henceforth *Ctnnb1*^{lox} and *Ctnnb1*$^{\Delta}$, respectively)[15]; *Ngn3-Cre* BAC transgenic mice [16] and the Cre-dependent EYFP reporter strain *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ [17]. *Pdx1-Cre*, *Pdx1-CreERT* [18] and *Elastase-CreERT* (*Ela-CreERT*) [19] transgenic mice were provided by Doug Melton (Harvard University). To induce recombination with *Pdx1-CreERT* or *Ela-CreERT*, we administered tamoxifen (Sigma) suspended in corn oil, by oral gavage. Embryos were genotyped by PCR, using primer sets described previously [6,9,18].

Tissue processing and staining

Pregnant dams were anesthetized with isoflurane and euthanized by cervical dislocation. Whole embryos (E13.5 and younger) and pancreata (E14.5 through adulthood) were dissected into ice-cold PBS for processing. Tissues were fixed overnight at room-temperature with zinc-buffered formalin (Polysciences) for paraffin sections or with 4% paraformaldehyde/PBS (2 hours-overnight at 4°C for frozen sections), and further processed as previously[3,8,20]. Series of duplicate paraffin sections (6 μm) were collected sequentially across multiple slides, spaced with skipping to span

the entire pancreas as described previously [3]. For labeling S-phase nuclei, mice were injected with BrdU (50 µg/g body weight) 1 hour prior to sacrifice.

Antibodies used for immunostaining are listed in Chapter 2 [3], and all secondary antibodies (raised in donkey) were purchased from Jackson Immunoresearch. Immunostaining was performed as described previously [3,8,20], including high-temperature antigen retrieval for paraffin sections. For anti-BrdU staining, frozen sections were pre-treated with DNAse I (700 u/ul, in 40 mM Tris-HCl pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$,10 mM $CaCl_2$) at room temperature for 30 minutes [21]. Bright field images were obtained using an Olympus CX41 microscope and MicroSuite software. For immunofluorescence, Fluoromount-G (Southern Biotech) was used as a mounting substrate and images were obtained using an Olympus IX71 microscope and MicroSuite software. Identical exposure times and postprocessing adjustments performed in Adobe Photoshop were used across control and experimental genotypes.

Quantification and analysis

To determine β-cell mass, pancreas size, and volume or number of cells expressing various markers, serial sections were stained by immunohistochemistry, and all sections on a single slide were photographed individually, to provide a representation of the entire pancreas. Cell counts, surface area, lineage tracing, and other quantification were performed using Photoshop count tool or ImageJ as previously described in Chapter 2 [3,8,20]. Calculations, graphs, and P-values (two-tailed, unpaired t-test) were generated in Microsoft Excel, and results are presented as mean +/- s.e.m.

Modeling pancreas growth

To model pancreas growth, we used the exponential growth equation $N(t)=N(0)*e^{(r*t)}$, where $N(t)$ represents the final population after a period of t days and $N(0)$ represents the initial population. The growth constant r, the rate at which the control pancreas expands over a day (r_{esc}), was calculated by substituting known values for pancreas size at E14.5 and E17.5 for $N(0)$ and $N(t)$, respectively. To determine the growth constant r for mutant cells (r_{mut}), r_{esc} was divided by the ratio of proliferation between control and mutant cells (BrdU labeling of control cells was 1.32-fold higher than that of mutant cells). The simulation was started at E14.5 using the known values of escaper (non-recombined) and mutant cells for the initial population ($N(0)$) of each group. Finally, the predicted number of escaper cells at day t was divided by the sum of the total predicted population of escapers and mutant cells. To model size, we used the growth constants generated for each population and back-calculated the percentage of escapers at E11.5, a point we previously determined to be unaffected by the loss of β-catenin [3] and started the simulation with both controls and PBsKO (described in Fig 1.1A) being the same.

Explant cultures and wholemount immunostaining

For ex vivo explant cultures, the dorsal buds of E11.5 pancreata were dissected in ice-cold sterile PBS, and cultured at the air-media interface for 3 days as described in Chapter 2 [3]. A small piece of tissue was collected for genotyping purposes at the time of dissection. β-catenin signaling-deficient explants (PBsKO) and their controls received no drug treatment. Pancreatic and kidney explants from E11.5 wild-type embryos were

treated with the PORCN inhibitor IWP-2 (Tocris 3533), 5 μM in DMSO, while controls received DMSO only treatment. Media was changed daily.

Wholemount immunofluorescence was performed as previously described in Chapter 2 [3,20]. Briefly, explants were fixed overnight in 4% PFA, washed, and stored in methanol until staining. For staining, explants were incubated in Dent's bleach for 2-4 hours, rehydrated to PBS, treated for 1 hour with PBS + 1% Triton-X100, and then placed in pre-block for 2 hours. Primary and secondary antibody incubations were performed overnight at room temperature. Confocal images were obtained using an Olympus FV-1000 microscope.

Results

β-catenin signaling regulates pancreas growth

Previously, we generated pancreas-specific β-catenin knockout mice (PBKO) [3,9] by deleting the conditional *Ctnnb1*^{lox} [15] allele with the *Pdx1-Cre*^{early} transgene (referred to here as *Pdx1-Cre*) [18], in mice also carrying the germline *Ctnnb1*$^{\Delta}$ [15] mutation, resulting in a pancreas devoid of any β-catenin function. To produce pancreas-specific β-catenin signaling-deficient mice (henceforth PBsKO), we likewise deleted *Ctnnb1*^{lox} with *Pdx1-Cre*, but replaced the germline null *Ctnnb1*$^{\Delta}$ allele with the germline β-catenin signaling-deficient allele *Ctnnb1*^{DM} [6](Fig. 3.1A). Similar to previous results from PBKO studies, PBsKO pancreata at the late fetal stage of E17.5 were much smaller than controls at the gross level (Fig. 3.1B-C). Our earlier characterization of PBKO mice provided clear evidence that β-catenin function is necessary for acinar cell development, and that a dramatic loss of exocrine tissue underlies the observed pancreatic hypoplasia [3,9,22]. By

using methods we developed to quantify absolute cell volume from stained sections, we found the total epithelial volume of PBsKO pancreata was reduced >3-fold at E17.5 compared to controls. This hypoplasia phenotype was less severe than that of PBKO, in which we previously reported a 10-fold reduction in size due to the loss of exocrine acinar cells (Fig. 3.1D). Likewise, β-cell volume was reduced ~1.3-fold in PBsKOs, a less severe phenotype than the ~2.3-fold reduction observed in PBKOs (Fig. 3.1E) [3]. Together these data indicate that the full measure of pancreas and β-cell mass requires the signaling function of β-catenin, but that nonsignaling roles of β-catenin may also contribute to pancreas growth.

Antibody staining identifies β-catenin signaling-deficient cells

Further characterization of PBsKO mice required the ability to distinguish β-catenin signaling-deficient cells from "escaper" cells that fail to undergo Cre-mediated recombination of *Ctnnb1*^{lox} and thus retain functional β-catenin protein. The C-terminal deletion engineered into the *Ctnnb1*^{DM}-encoded protein (henceforth, βCAT^{DM}) eliminates binding of C-terminus specific antibodies [β-cat(C-term)], whereas antibodies designed against the central armadillo protein core [β-cat(ARM)] will bind both βCAT^{DM} and βCAT^{WT} [6]. We stained control and PBsKO pancreata with both β-cat(ARM) and β-cat(C-term), along with anti-E-cadherin to label the entire epithelium (Fig 3.2A-L). As expected, control pancreata were stained with both β-cat(ARM) and β-cat(C-term), indicating that the antibodies can simultaneously recognize separate regions of the β-

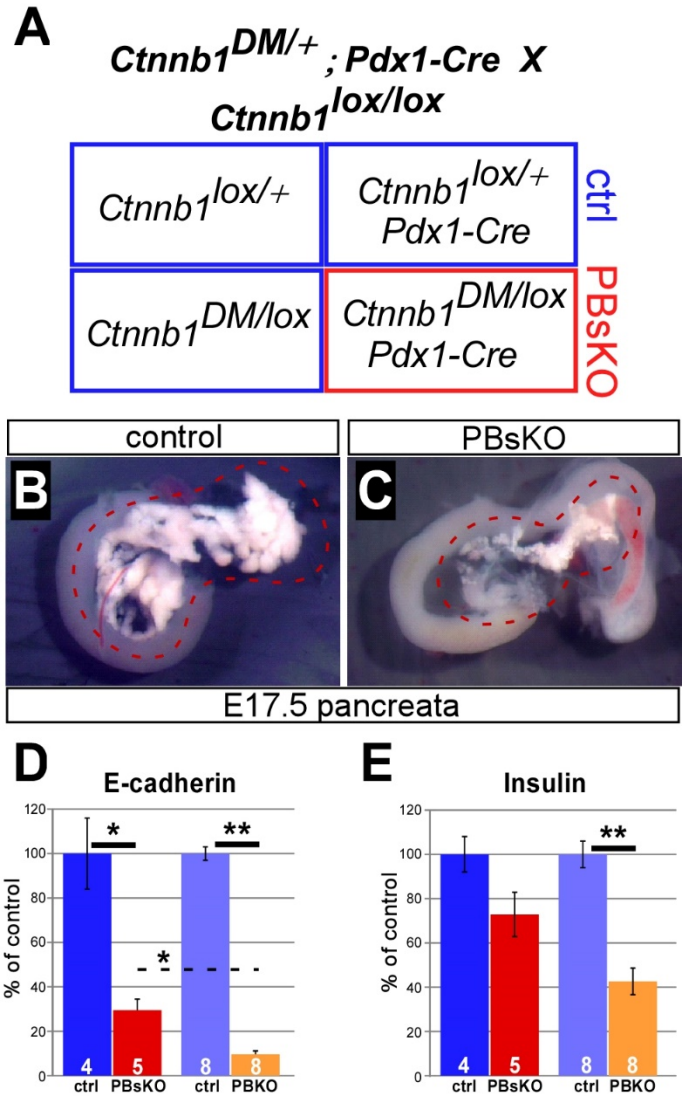

Figure 3.1. β-catenin signaling is required for embryonic pancreas growth. PBsKO pancreata were analyzed and compared to controls at E17.5. **(A)** Breeding scheme to generate control and PBsKO genotypes. **(B-C)** Images of E17.5 control and PBsKO pancreata reveal a reduced pancreatic size in PBsKOs. **(D)** Pancreata that were stained with E-cadherin (not pictured) were quantified and normalized to controls, and exhibit a 3.4-fold reduction in PBsKO compared to controls, whereas PBKO were 10-fold smaller than controls. **(E)** Quantification of Insulin+ β-cells normalized to controls at E17.5 reveals a more severe reduction of β-cell mass in PBKO than PBsKO when compared to littermate controls. The number of pancreata analyzed per condition is listed in the bottom of each column. Error bars are s.e.m.

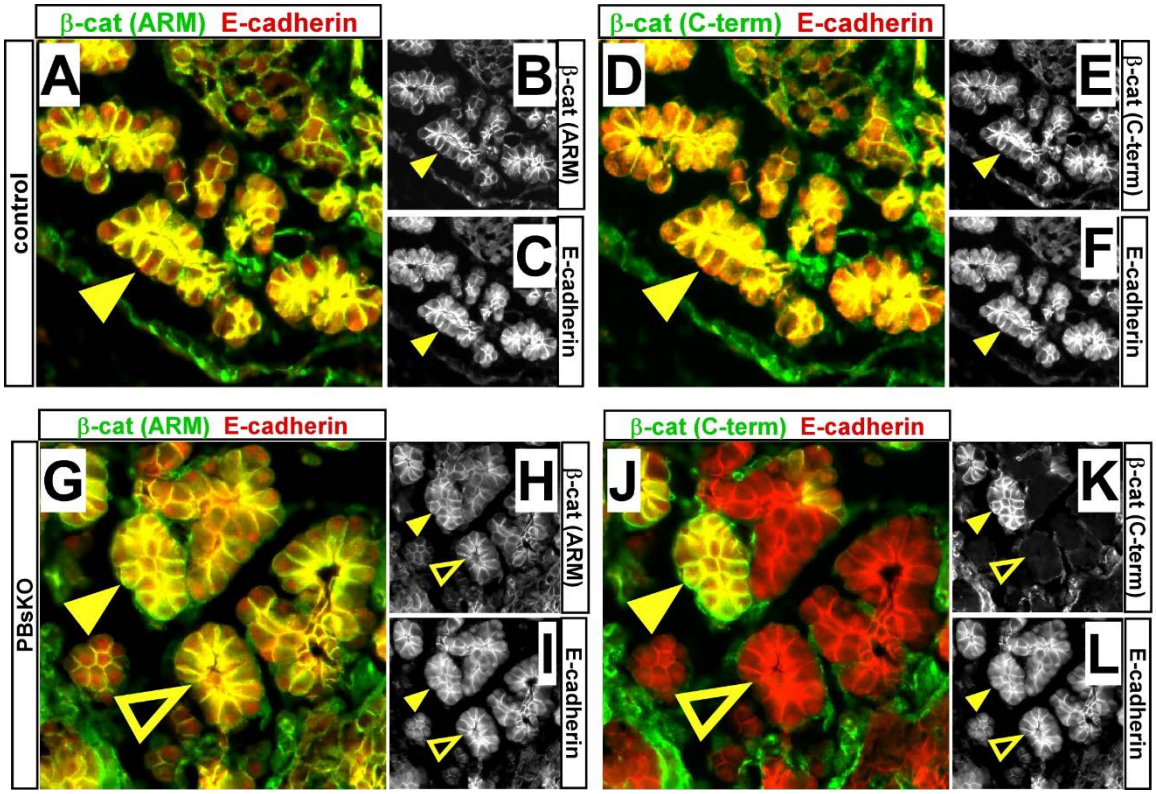

Figure 3.2. Antibody staining identifies β-catenin signaling-deficient cells. E14.5 control and PBsKO pancreata were stained with antibodies that recognize either the central armadillo core (ARM) or the C-terminus (C-term) of β-catenin. **(A-C)** As expected, all epithelial cells from control pancreata stained for E-cadherin (red) and for full length β-catenin (green). **(D-F)** The same field as in (A) is shown stained for β-catenin (C-term) which clearly labels all epithelial cells. **(G-I)** All epithelial cells are co-labeled by the β-catenin (ARM) antibody in PBsKO. **(J-L)** Potential β-catenin signaling-deficient cells are identified by their lack of β-catenin (C-term) staining, shown with the open yellow arrowhead. The closed arrowhead indicates "escaper" cells that retain a functional copy of β-catenin stain with both antibodies.

catenin protein (Fig. 3.2A-F). In PBsKO mice, we found that all epithelial cells stained with β-cat(ARM) (Fig 3.2G-I), however, numerous clusters of pancreatic epithelial cells failed to co-stain with β-cat(C-term) (Fig 3.2J-L), indicating expression of βCAT^{DM} but not βCAT^{WT}. Therefore, we interpret β-cat(C-term)-negative cells to be β-catenin signaling-deficient cells of the pancreatic epithelium.

Functional association of signaling-deficient
β-catenin with adherens junctions

In addition to the canonical role of β-catenin in Wnt signaling, β-catenin also stabilizes adherens junctions in the pancreas where it associates with E-cadherin and α-catenin. In the absence of β-catenin function, E-cadherin junctions appear unchanged in the pancreas [8,9]. Previous work from our lab and others indicates that another catenin family member, plakoglobin (γ-catenin), can act redundantly and stabilize junctions in the absence of β-catenin [9,23]. We tested whether the truncated βCAT^{DM} protein was still found at E-cadherin-associated junctions, or whether it was replaced by plakoglobin as in PBKO. We stained E11.5 PBKO and control pancreata for E-cadherin and plakoglobin and, confirming our previous report, observed strong co-localization of E-cadherin and plakoglobin at the cell membrane in PBKO but not controls (Fig. 3.3A-F). To determine if plakoglobin was similarly localized at the cell membrane in PBsKO, we stained E11.5 pancreata with β-cat(C-term) and plakoglobin and observed, in contrast to the PBKO phenotype, no detectable increase in plakoglobin staining (Fig. 3.3G-L). Therefore, we infer that structural aspects of β-catenin function are retained in β-catenin signaling-deficient cells as shown previously in mouse and *Drosophila* [6]. Furthermore, our results

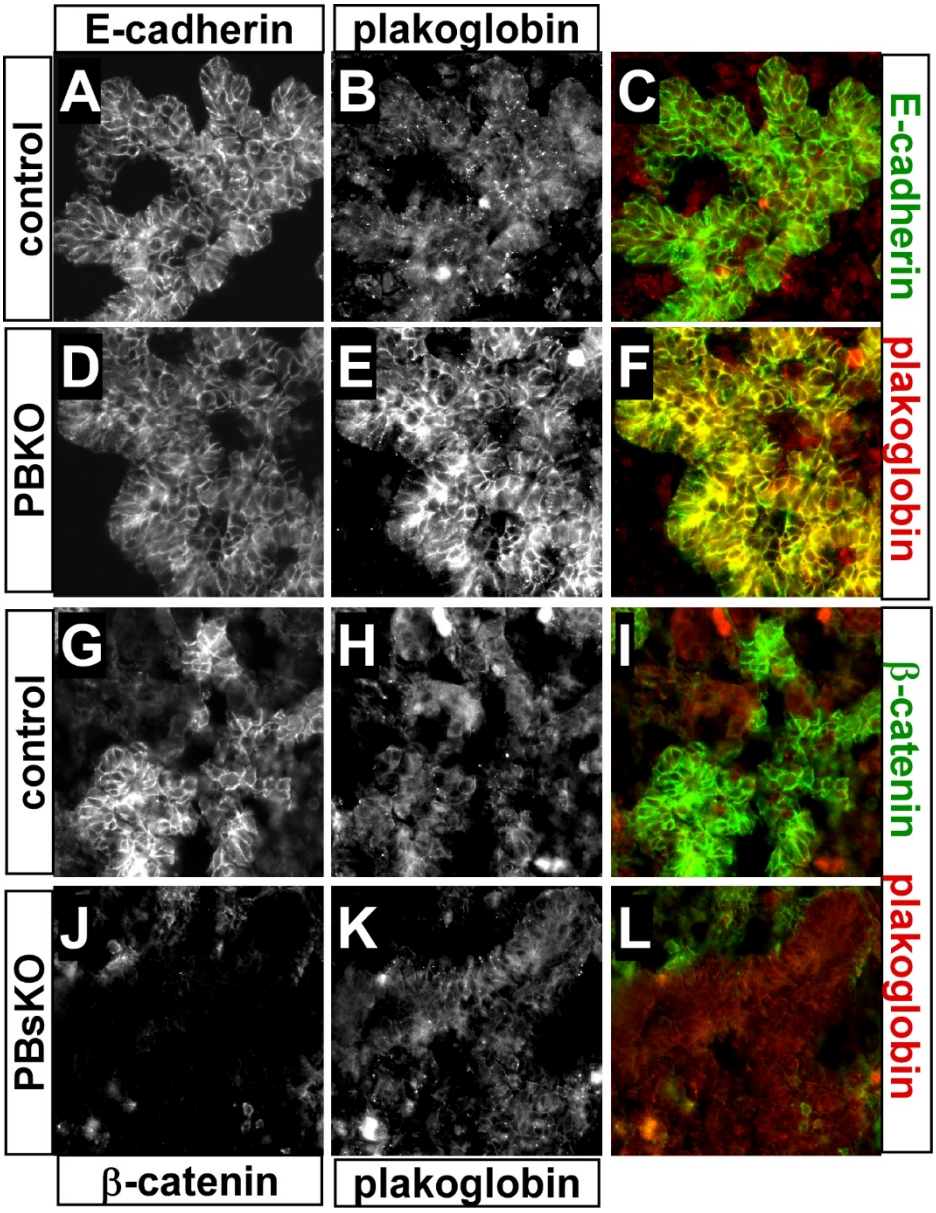

Figure 3.3. βCAT^{DM} associates with adherens junctions. Plakoglobin staining was performed on E11.5 control, PBKO, and PBsKO pancreata with E-cadherin or β-catenin. **(A-C)** Adherens junctions of E11.5 control pancreatic epithelium stain for E-cadherin but not plakoglobin. **(D-F)** Robust junctional plakoglobin staining is found in PBKO epithelium, potentially replacing β-catenin. **(G-I)** All control cells stain for β-catenin (C-term) but not plakoglobin at the junctions. **(J-L)** PBsKO pancreata do not stain for plakoglobin or β-catenin (C-term).

indicate that plakoglobin upregulation in the absence of β-catenin reflects a "sensing" mechanism operating at the adherens junction, rather than a signaling-dependent feedback mechanism.

β-catenin signaling is not essential for acinar cell development

Because epithelial and β-cell volume were not as diminished in PBsKO pancreata compared to PBKO pancreata, we were particularly interested in determining whether PBKO associated acinar loss also occurs in the PBsKO pancreas. To examine acinar cell development in the absence of β-catenin signaling, we stained late fetal stage PBsKO acinar cells for the digestive enzyme amylase and for β-cat(C-term) to distinguish escaper cells from potential signaling-deficient acinar cells. All control acinar cells stained for β-cat(C-term) as anticipated (Fig. 3.4A-C). However, we were surprised to find found numerous acinar cell lobules in PBsKO pancreata that were devoid of β-cat(C-term) staining (Fig 3.4 D-F). This result was in stark contrast to PBKO tissue, in which nearly all acinar cells that develop represent escaper cells that did not delete *Ctnnb1*$^{lox\,9}$. These observations were confirmed by quantifying the fraction of escaper vs. true knockout acini (βCAT^{WT}-negative) in PBKO and PBsKO tissues. We found that 96% of all acini in PBKO were escapers, compared to only 44% in PBsKO (Fig 3.4G). Therefore, we conclude that β-catenin signaling is not essential for acinar differentiation. The fact that escaper βCAT^{WT} cells do contribute to over half of acinar cells, however, implies a selective disadvantage of β-catenin signaling-deficient cells, potentially at the level of proliferation.

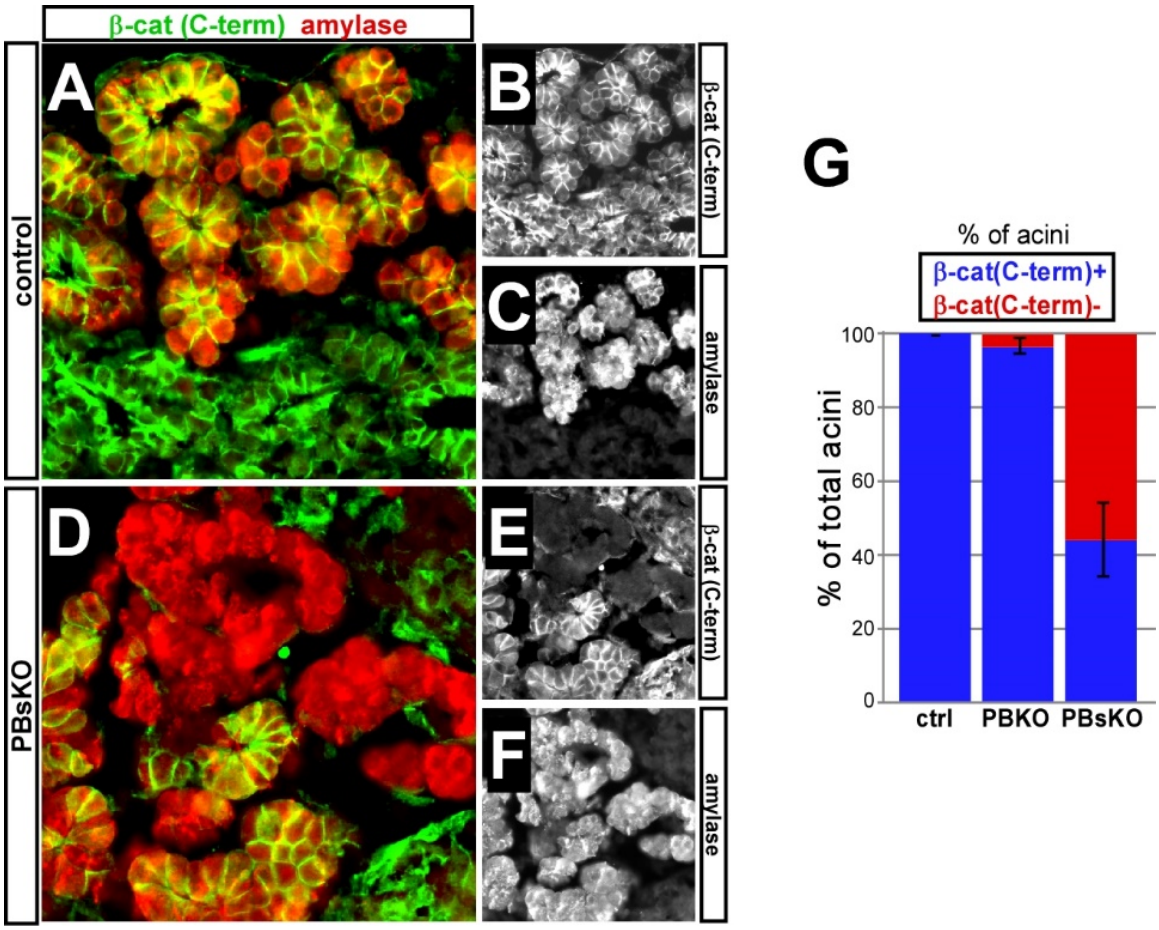

Figure 3.4. Acinar cells develop in the absence of β-catenin signaling. E17.5 pancreata were stained with a β-catenin (C-term) antibody (green) and the digestive enzyme amylase (red) to identify potential β-catenin signaling-deficient acinar cells. **(A-C)** Acinar cells, juxtaposed to islets (green), from control pancreata co-stain with β-catenin and amylase. **(D-F)** Amylase+ β-catenin (C-term)- acinar cells develop in PBsKO pancreata at E17.5, along with β-catenin (C-term)+ "escaper" acinar cells. **(G)** The percentage of β-catenin (C-terminal)+/- acini was calculated in PBKOs and PBsKOs and revealed that most acini in PBKOs were escapers, whereas less than half of all acini in PBsKO were escapers.

β-catenin signaling regulates proliferation in distal progenitors and acinar cells

We and others previously found that β-catenin regulates proliferation of pancreatic progenitors during development and acinar cells during postnatal expansion, homeostasis, and regeneration, whereas it is not required for β-cell replication [3,8,9,22,24]. Considering these results, we sought to understand whether the signaling function of β-catenin specifically promotes the proliferation of progenitors, acinar precursors, and early acinar cells. To examine the potential requirement for β-catenin signaling in proliferation, we injected pregnant mice with BrdU to label proliferating cells 1 hour prior to harvest at E12.5 and E14.5, and analyzed BrdU labeling of Cpa1+ (carboxypeptidase 1) MPCs at E12.5 (not shown), or early acinar cells at E14.5 (Fig 3.5A-F). For the postnatal day 7 (P7) timepoint, we identified proliferating acinar cells by immunofluorescence for Ki67 and the digestive enzyme amylase (images not shown). Because we used different methods to assay proliferation, we chose to normalize each period to the control population of each given time point. We found that at E12.5, E14.5, and P7, control cells consistently proliferated at a rate ~1.3-fold higher that of PBsKO cells (Fig. 3.5G). Again, this difference was less severe than that observed found in PBKO, where we found a ~2-fold reduction in proliferation [3]. These results confirmed that β-catenin signaling is required for the full extent of pancreas growth. However, it is worth noting that in the absence of β-catenin signaling, a significant level of proliferation still occurs, yet the PBsKO pancreas remained hypoplastic compared to controls.

Given the change in proliferation between control and signaling-deficient cells, we predicted that, similar to PBKO, escaper cells would eventually overtake the PBsKO

pancreas. Using the same strategy as in Figure 3.4G, we calculated the fraction of escaper cells found at two additional time points, E14.5 and P7, and found that βCAT^{WT}+ escapers gradually but steadily overtake mutant cells by P7, eventually making up the majority of existing acinar cells (Fig. 3.5H, blue line). Though a clear trend toward escaper take-over was evident from these data, we sought to independently verify that the difference in proliferation between escapers and mutant cells could account for our observation by simulating pancreas growth with a simple mathematical model (see Materials and Methods and Fig 3.6). We used the exponential growth equation, $N(t) = N(0) * e^{(r*t)}$ to predict the rate at which escapers would overtake the acinar population. The forecasted escaper percentage tracked closely with empirical data, predicting that the majority of acinar cells would be escapers by the postnatal period (Fig. 3.5H, orange line). We also asked whether the difference in proliferation could explain the size difference observed at E17.5, when the PBsKO pancreas is ~3.4-fold smaller than controls. We modeled pancreas growth from E11.5, a point previously determined to be relatively unchanged in β-catenin mutants [3], and found that the predicted size difference followed closely with our observations (Fig. 3.5I). In each model, the growth equation lagged slightly behind the actual differences in escaper percentage and size, reflecting a potential limitation of these data. Nonetheless, these simple mathematical models support our empirical data and demonstrate the signficant consequences to organ growth of relatively limited changes in proliferation.

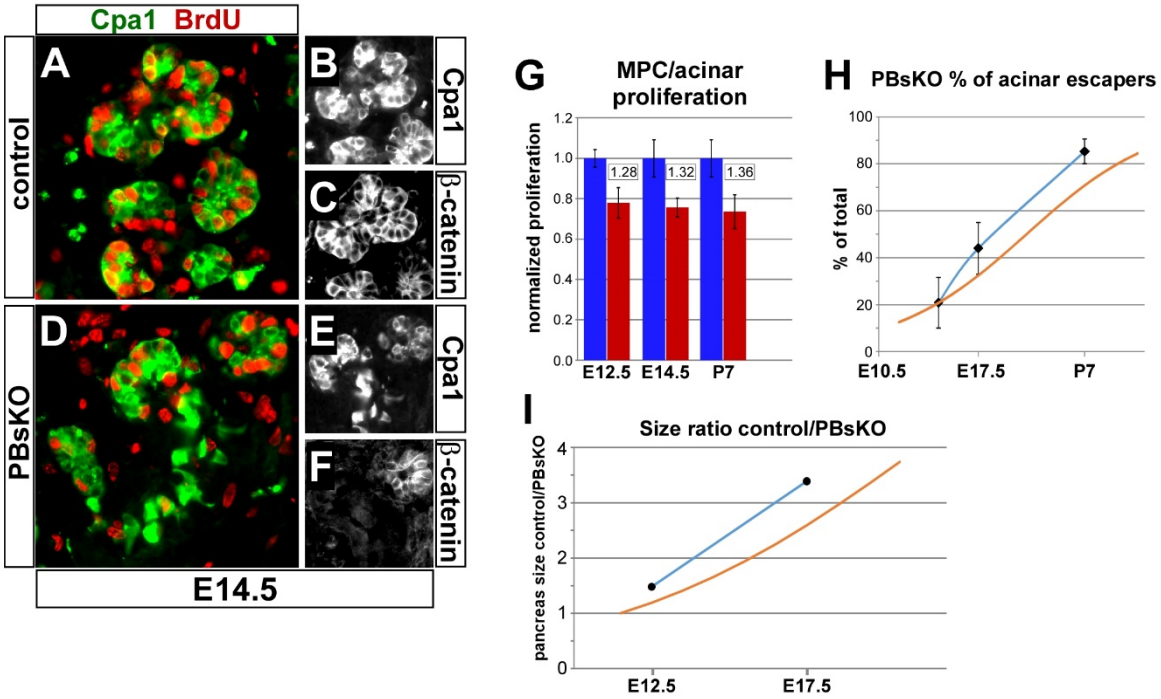

Figure 3.5. β-catenin signaling regulates proliferation in MPCs and acinar cells. Control and PBsKO littermates received BrdU 1 hour prior to sacrifice at E12.5 and E14.5, while P7 pancreata were stained for Ki67, and proliferation index was assessed and normalized to controls. Proliferating cells are labeled by BrdU or Ki67 immunostaining (red) and Cpa1 marks distal-tips (green). The absence of β-catenin (C-term) immunostaining (i.e., panel F) was used to identify PBsKO cells. **(A-C)** Abundant BrdU+ Cpa1+ control cells were all co-labeled with β-catenin at E14.5. **(D-F)** Fewer Cpa1+ BrdU+ distal tip cells are found in E14.5 PBsKO pancreata in β-catenin (C-term)- cells. **(G)** The proportion of proliferating cells normalized to controls is shown at E12.5, E14.5, and P7. PBsKO cells proliferate at a rate ~1.3-less than controls at all assayed time points. **(H)** The percentage of distal-tip/acinar cells that were escapers in PBsKOs is shown at E14.5, E17.5, and P7 (blue line), revealing a gradual takeover by βCAT^{WT}+ cells as development progresses. A mathematical simulation of escaper versus mutant cell percentage is shown plotted on the same graph (orange line) beginning at E14.5 where the percentage of escapers was known, indicating the likely takeover of escapers. **(I)** The size difference ratio between controls and PBsKO pancreata is shown at E12.5 and E17.5 (black dots and blue line). The predicted size difference (orange line) between controls and PBsKO closely tracks with observed data.

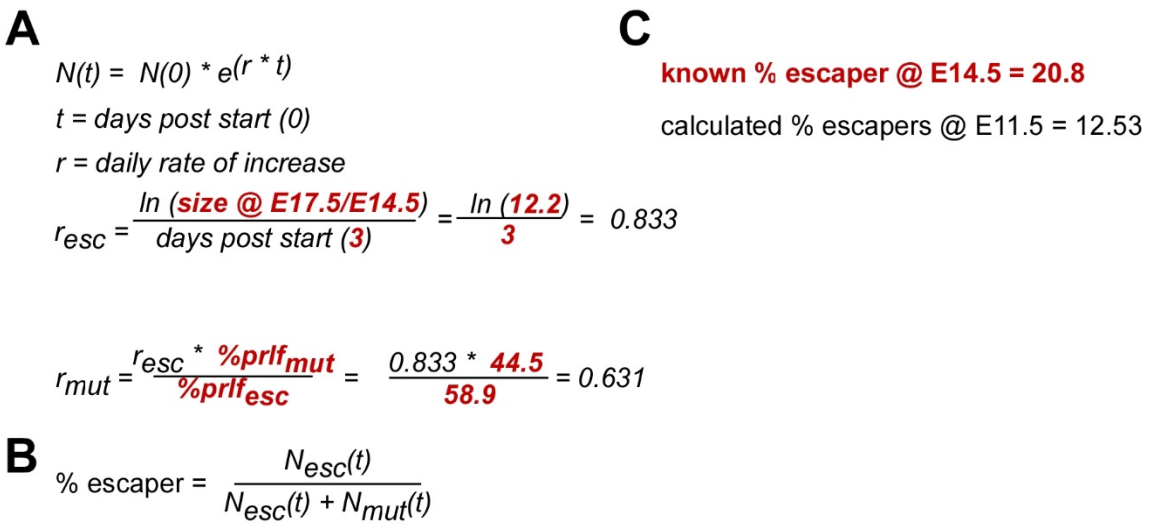

Figure 3.6. Modeling PBsKO development using exponential growth. An exponential growth formula was used to model PBsKO pancreas growth given the co-existence of escaper and mutant cells within the pancreatic epithelium. **(A)** The exponential growth formula that was used to model growth is shown along with the definitions of each variable. We calculated the growth rate constants by utilizing empirical data (shown in bold red lettering) we previously obtained in other experiments. **(B)** The percentage of escapers was calculated by dividing their predicted number by the total predicted PBsKO pancreas size at *t* days. **(C)** Because β-catenin has little effect on the E11.5 pancreas, size was modeled by predicting the number of escapers present at E11.5 by using known data from E14.5 (shown in red lettering), and simulating PBsKO and wild-type growth.

β-catenin signaling regulates postnatal acinar cell expansion

Previous studies demonstrate that β-catenin is critical for postnatal acinar cell proliferation and for acinar cell regeneration [8]. To determine whether acinar cell proliferation was attributable to β-catenin signaling, we generated acinar specific β-catenin signaling-deficient mice (ABsKO) using similar breeding schemes as described earlier, substituting the tamoxifen mediated acinar-specific *Elastase-CreERT* transgene [19] for the *Pdx1-Cre* transgene. Importantly, the mice resulting from these crosses all carry the Cre-dependent $R26R^{EYFP}$ reporter [17], allowing for lineage tracing of recombined cells. To delete β-catenin signaling and simultaneously activate the $R26R^{EYFP}$ reporter, we gave tamoxifen to nursing mothers at birth (P0) and analyzed EYFP labeling 7 or 30 days after tamoxifen administration. Additionally, to assess proliferation, we administered BrdU 1 hour prior to sacrificing mice at P7 or P30. At P7, ABsKO mice exhibited a significant reduction in the number of BrdU+ EYFP+ acinar cells as compared to controls (Fig. 3.7A-E). This result is consistent with previously published results obtained from deleting all β-catenin function in acinar cells [8], and support a role for β-catenin signaling in juvenile acinar cell proliferation. The decrease in proliferation rate detected by BrdU incorporation, in P7 ABsKO mice, appears greater than that previously observed using Ki67 staining of P7 PBsKO mice (Fig 3.5G). We attribute this discrepancy to the different methods used to identify proliferating cells, BrdU labeling being strictly specific to cells progressing through S-phase while Ki67 is expressed during the entire cell cycle and could persist in cells that are not progressing through S-phase due to lack of β-catenin signaling. After the postnatal period, at P30, the percentage of EYFP+ acinar cells was significantly decreased from that observed in controls, consistent with a

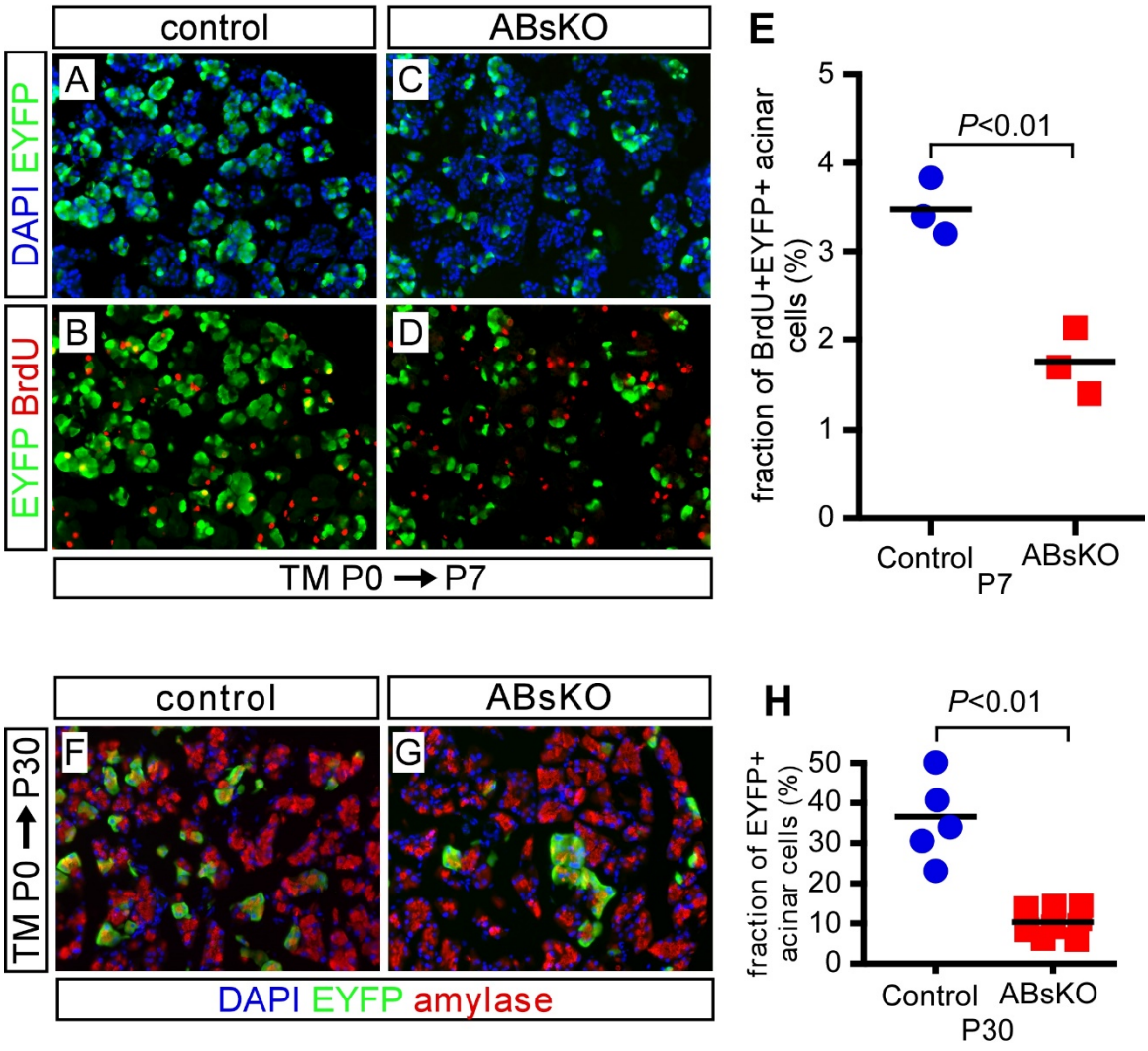

Figure 3.7. β-catenin signaling is critical for acinar cell proliferation during postnatal growth. (A-D) P7 mice were pulsed with tamoxifen on the day of birth, and received a 1-hour pulse of BrdU prior to sacrifice to label proliferating acinar cells. Immunofluorescent staining for EYFP+ acinar cells (green) and DAPI stained nuclei (blue) labeled by the S-phase marker BrdU (red). The percentage of proliferating EYFP+ BrdU+ cells is decreased in ABsKO mice compared to controls. **(E)** Quantification of the BrdU labeling index of EYFP+ cells reveals a significant reduction in the proliferative capacity of β-catenin signaling deficient acini. **(F-G)** Postnatal day 30 (P30) control or ABsKO mice received tamoxifen on the day of birth to induce recombination. Staining for the EYFP lineage (green) of amylase+ acinar cells (red) reveals fewer EYFP+ cells in ABsKO pancreata at P30, compared with controls. **(H)** The percentage of EYFP labeling of amylase+ acinar cells is decreased in ABsKO mice at P30, indicating that β-catenin signaling deficient acinar cells do not efficiently contribute to postnatal expansion of the exocrine pancreas (used with permission from Matthew Keefe).

proliferative disadvantage favoring the expansion of EYFP-negative escaper cells (Fig. 3.7F-H). These data strongly indicate that the signaling function of β-catenin is critical for expansion of distal MPCs as well as mature acinar cells, but is likely dispensable for the establishment and maintenance of this lineage.

β-catenin structural function is partially sufficient for distal-proximal patterning

In Chapter 2 [3], I demonstrated that loss of β-cell mass in PBKO pancreata was not due to a defect in the differentiation of endocrine precursors, but instead correlated with an early and specific loss of distal MPCs. This result revealed a novel role for β-catenin, in maintaining proximal–distal patterning of the early epithelium, as distal MPCs resorted to a proximal, endocrine-competent "trunk" fate following β-catenin deletion. In addition to a quantitative reduction in total β-cell mass, the loss of MPCs in the PBKO pancreas produces a qualitative, near-total depletion of acinar cells. As acinar cells still developed in PBsKO, we hypothesized that distal MPCs would persist in the absence of β-catenin signaling function, albeit in a context of reduced proliferation and growth.

To determine whether distal-proximal patterning is maintained in PBsKO pancreata, we stained and counted putative E12.5 MPCs, labeled by the distal-tip-specific marker Ptf1a [14] (Fig 3.8A-B). We found that although Ptf1a+ MPCs were reduced in PBsKO compared to controls, their numbers were significantly increased compared to PBKO [3] (Fig 3.8C). Much of the reduction of MPCs that is observed, in PBsKO, is likely due to the overall reduction in epithelial volume, which is similar between PBsKO and PBKOs (Fig. 3.8D). Consistent with preserved patterning in the absence of Wnt/β-

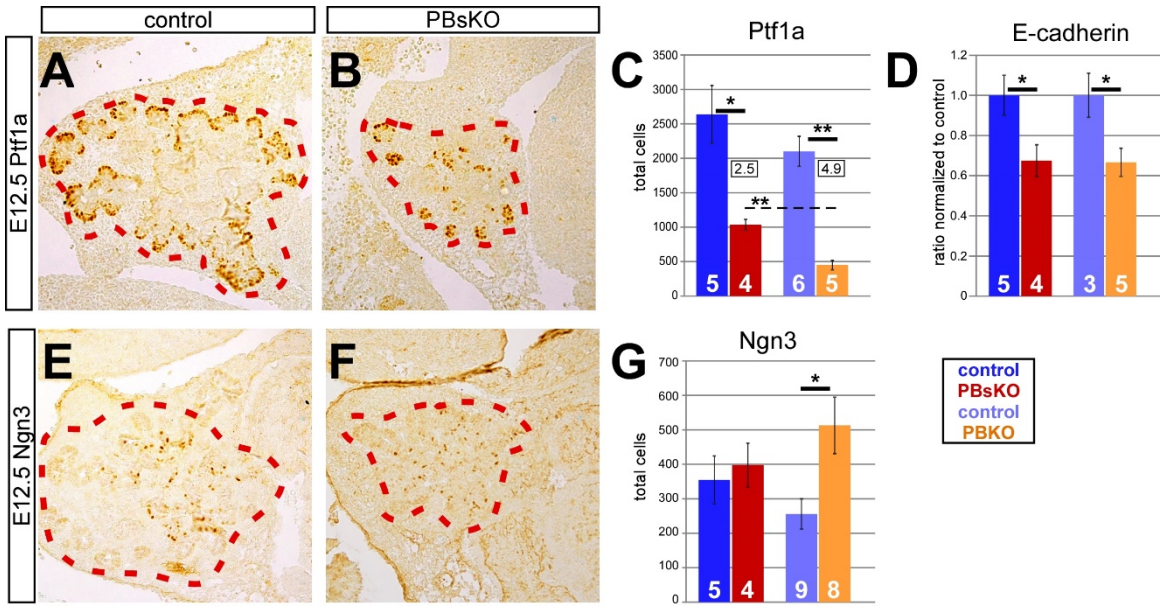

Figure 3.8. Patterning is maintained in early PBsKOs. E12.5 mutant and control pancreata were stained for Ptf1a to label distal MPCs, E-cadherin to label all epithelial cells, and Ngn3 to label proximal endocrine precursors. The pancreatic epithelia is outlined in red in the images. (A-B) Controls stained for Ptf1a pinpoint MPCs localized to the distal periphery of the pancreatic epithelium. Fewer Ptf1a+ cells were found in PBsKOs compared to controls. (C) Quantification of Ptf1a+ cells in controls, PBsKO, and PBKO revealed a significant loss of Ptf1a+ cells in mutants compared to controls. However, the drop in Ptf1a+ cells in PBsKO is less severe than in PBKOs. (D) The size of pancreatic epithelium in PBsKO and PBKO are similarly reduced compared to controls at E12.5. (E-F) Control and PBsKO pancreata stained for the endocrine precursor Ngn3, revealed little difference in Ngn3+ staining. (G) No difference in the number of Ngn3+ cells was found between controls and PBsKO. In PBKOs we previously observed a significant increase of Ngn3+ cells compared to controls [3], suggesting that patterning is maintained in the absence of β-catenin signaling.

catenin signaling, Ptf1a+ MPCs are found to localize normally to the periphery of the branched epithelium in PBsKO pancreata (Fig. 3.8B). Together, these data suggest that restoring the structural function of β-catenin can rescue MPC patterning defects found in PBKO. We previously found that Ngn3+ pre-endocrine precursors were approximately doubled in number in PBKO pancreata at E12.5 [3]. By contrast, we found no significant increase in Ngn3+ cells in PBsKO, compared to controls (Fig. 3.8E-G). Together with the more tempered loss of Ptf1a+ cells, we interpret these data to indicate that non-signaling roles of β-catenin make a critical contribution to early tip-trunk patterning in the developing pancreas.

Wnt ligands are not necessary for the differentiation
of acinar or endocrine cells

While the source and variety of Wnt ligands involved in pancreas development are not known [7], our findings would suggest that canonical β-catenin-mediated Wnt ligands should be dispensable for acinar cell development and patterning but important for epithelial expansion. To determine whether Wnt ligands are required to maintain patterning, we cultured wild-type E11.5 pancreata in the absence or presence of the Porcn inhibitor IWP-2 [25]. Porcn is required for secretion and bioactivity of all known Wnt ligands [26,27], and its inhibition with IWP-2 in metanephric kidney explants resulted in loss of Wnt/β-catenin-dependent branching morphogenesis [28]. We recapitulated this result using E11.5 kidney explants treated with 5 μM IWP-2, confirming the efficacy of this small molecule as a tool to inhibit Wnt signaling in pancreatic explant culture (Fig. 3.9). Dorsal pancreatic bud explants were grown for 3 days in the absence or presence

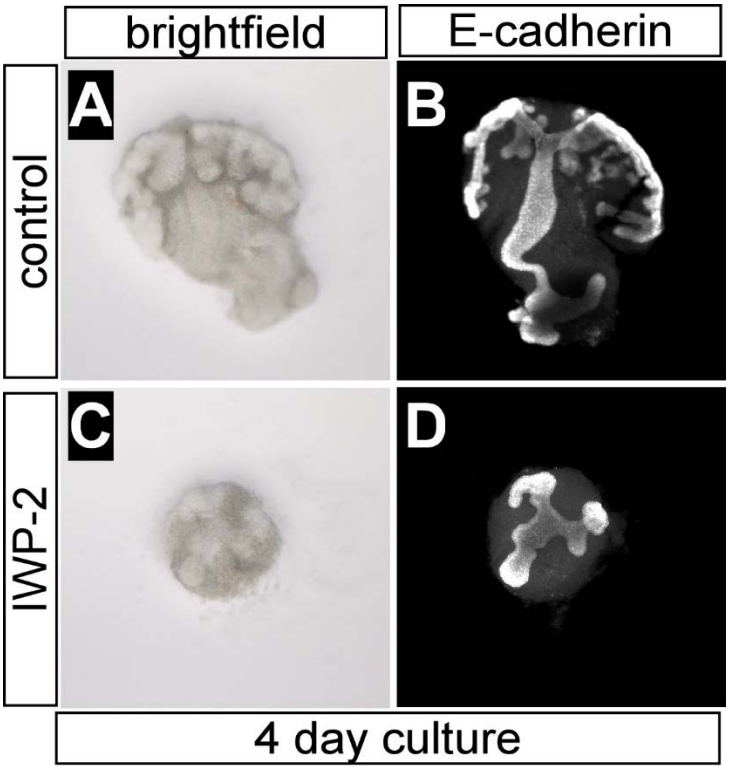

Figure 3.9. IWP-2 blocks branching in kidney explants. To confirm the efficacy of IWP-2 as a Wnt inhibitor for pancreas explant cultures, E11.5 wild-type kidney explants were grown for 4 days in the absence or presence of 5 μM IWP-2. Explants were imaged by bright field imaging and through immunofluorescent staining of E-cadherin to label the epithelial branches. **(A-B)** Control kidneys grew and readily branched into the surrounding kidney mesenchyme. **(C-D)** IWP-2 treated kidneys branching was severely inhibited, confirming previous reports and indicating the efficacy of this drug.

of IWP-2, and stained for the tip/acinar marker Cpa1 and the α-cell marker glucagon. We previously demonstrated that β-catenin-deficient pancreatic epithelia do not generate acinar cells under these culture conditions [3]. By contrast, we found that both control and IWP-2-treated explants developed Cpa1+ acinar cells as well as glucagon+ cells (Fig. 3.10A-B). This in vitro result supports our conclusion, based on PBsKO phenotypes in vivo, that the canonical Wnt/β-catenin signaling pathway is not required to maintain MPCs or differentiate acinar cells.

To directly determine the effects of blocking β-catenin signaling in this explant culture system, we cultured control and PBsKO explants as above. As expected, abundant Cpa1+ acinar cells developed in both control and PBsKO explants; importantly, the vast majority of these arose in the latter from βCAT^{DM}+ progenitors, whereas Cpa1+ cells developing in PBKO explants predominantly arose from βCAT^{WT}+ escapers (Fig. 3.10C-I). By eliminating either Wnt ligand production or β-catenin signaling activity, and observing similar phenotypes, our findings indicate that Wnt/β-catenin signaling is largely dispensable for pancreatic patterning and acinar cell differentiation, whereas β-catenin structural function plays a critical role in these processes.

Discussion

Previously, we and others found that acinar cell development, postnatal expansion, and acinar cell regeneration, but not β-cell differentiation and function, were dependent on the multifunctional protein, β-catenin [8,9,22]. Furthermore, in Chapter 2 of this dissertation, I demonstrated that β-catenin is required prior to endocrine specification to establish normal islet cell number in the pancreas [3]. We attributed this requirement to

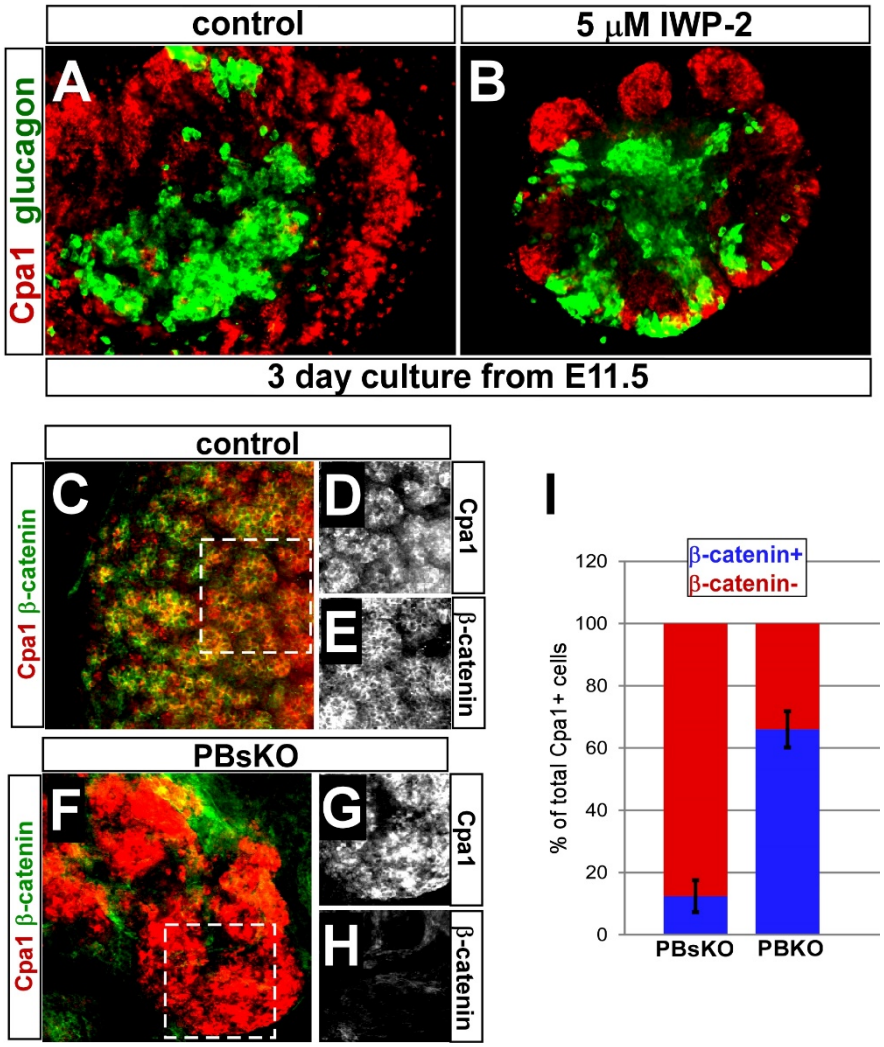

Figure 3.10. Patterning is maintained in the absence of Wnt signaling ex vivo. **(A-B)** Wild-type E11.5 dorsal pancreatic buds were cultured for 3 days with or without the Porcn inhibitor IWP-2 (5 μM). IWP-2 treatment reduced overall explant size, but did not perturb the normal proportions of Cpa1+ (red) and glucagon+ (green) cells. **(C-H)** Control or PBsKO dorsal bud explants were cultured for 3 days, and stained for Cpa1 (red) and β-catenin (C-terminal) (green). The majority of Cpa1+ cells in PBsKO explants lack staining for the β-catenin C-terminus (i.e., express βCAT^{DM} rather than βCAT^{WT}), indicating that β-catenin signaling is not required ex vivo for patterning or acinar cell development. **(I)** Quantification of the percentage of β-catenin (C-term)+ acinar cells in PBsKO and PBKO. Together, these data indicate that Wnt/β-catenin signaling is not required for acinar cell development ex vivo.

two distinct roles for β-catenin during pancreas development: first, it promotes and maintains distal-proximal patterning of the epithelium by inhibiting Notch function, and second, it is required for the full measure of proliferation in the early epithelium [3]. However, these studies could not determine whether the two distinct roles for β-catenin, patterning and proliferation, were mediated by its signaling or structural functions. In the present study, we re-address the role of β-catenin in pancreas development by utilizing a signaling-deficient, yet structurally competent allele of β-catenin (*Ctnnb1*^{DM}) [6]. We infer that the signaling and structural functions of β-catenin are required separately for the maximal proliferation and growth of the organ, and to maintain distal-proximal patterning and acinar cell differentiation, respectively. The evidence presented in this chapter offers a new perspective of β-catenin during pancreas development, assigning the signaling and structural roles of the protein to proliferation and patterning, respectively (Fig. 3.11).

Whereas prior studies eliminating both the Wnt signaling and structural function of β-catenin just after specification (PBKO) resulted in the loss of acinar cell differentiation and expansion [9,22] inhibition of Wnt ligand signaling, through the expression of a soluble, dominant-negative Frizzled8 Wnt receptor, impeded proliferation but did not prevent acinar cell differentiation or pancreatic function [29]. Moreover, deleting β-catenin from the pancreatic epithelium beginning several days after specification, when many MPCs are already acinar restricted, does not prevent acinar cell development, but does negatively affect proliferation [24]. Previously, we also found that acinar-specific deletion of β-catenin after birth does not alter functionality or acinar cell identity, but is required for regeneration following injury and for postnatal expansion,

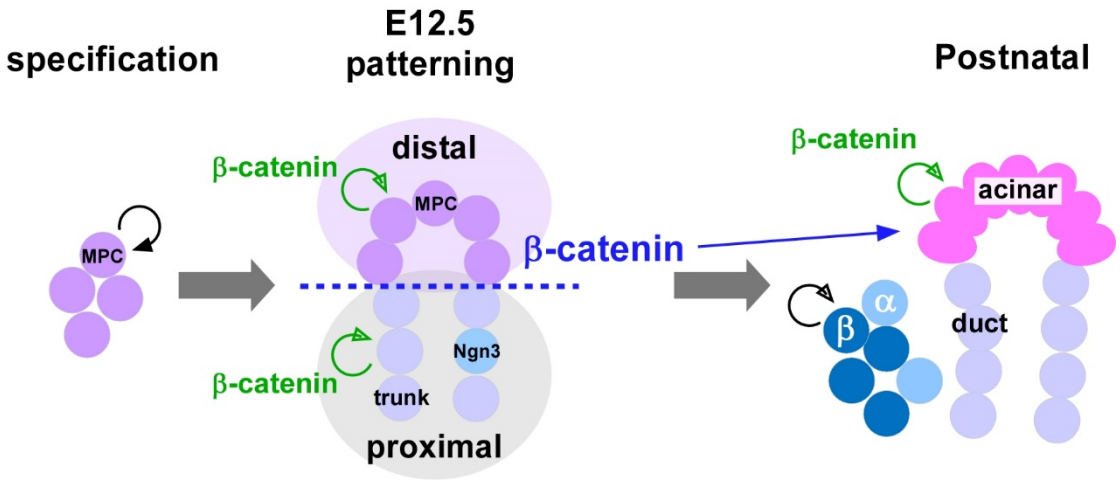

Figure 3.11. Multiple roles for β-catenin during early pancreas development. Following specification and prior to E12.5, MPCs (purple) are relatively unorganized, until a dramatic shift in morphology occurs referred to here as patterning. After E12.5 MPCs are found at the distal-tip of the pancreatic epithelium (shaded purple) and proximal-trunks (shaded grey) comprise trunk progenitors and Ngn3+ endocrine precursors. A new role for β-catenin in maintaining the distal-proximal patterning of early pancreatic progenitors has been identified, indicated by the blue dashed line between the two domains at E12.5. Evidence suggests that the structural role of β-catenin (blue lettering/arrows) mediates this process. The signaling role of β-catenin (green lettering/arrows) is required for pancreatic growth and proliferation of the distal and proximal cells during early phases of development and during acinar postnatal acinar cell expansion. Black arrows indicate proliferation occurring independent of β-catenin.

supporting a progrowth role for β-catenin in acinar cells [8]. These data suggest that, once cell fates are committed, acinar cell development proceeds independent of β-catenin, but that maximal proliferation requires β-catenin. In Chapter 2, I showed that the loss of acinar cells in PBKO is reflected by an early and dramatic loss of uncommitted MPCs and disrupted distal-proximal patterning [3]. Here, we provide direct evidence showing that the Wnt signaling function of β-catenin is dispensable for acinar cell development but is required for maximal proliferation in utero and during the postnatal period. Furthermore, we found that distal-proximal patterning in PBsKO pancreata was maintained compared to that observed in PBKOs. Nonetheless, there were fewer Ptf1a+ distal cells in PBsKO compared to control, which we attribute to the loss in proliferation and the natural transition from MPC to bi-potent trunk progenitor. Finally, the reduction of β-cell mass in PBsKO was less dramatic than that observed in PBKO. We believe that the combined disruption of patterning and proliferation aggravates the β-cell development defect in PBKO pancreata, likely secondary to the dramatic loss of MPCs, whereas in PBsKO pancreata, the reduction in β-cell mass is due primarily to decreased proliferation of otherwise normally patterned progenitor cells.

Decades-old studies of rodent ex vivo pancreas development have emphasized the importance of extrinsic signals originating from pancreatic mesenchyme for exocrine growth [30,31]. Coupled with the obvious defects in the exocrine pancreas in the absence of β-catenin [9,22], we speculated that Wnts from pancreatic mesenchyme are necessary for distal-acinar cell growth. However, eliminating all Wnt ligands genetically is complicated by redundancy and all potential sources of Wnts have not been identified during pancreas development [7]. The acyltransferase *Porcn* is necessary for the secretion

and bioactivity of all Wnt ligands, such that eliminating its activity results in the loss of all Wnt ligand activity [26,27]. To evade the aforementioned issues with eliminating Wnt ligands genetically, we cultured pancreatic explants ex vivo in the presence of the Porcn inhibitor IWP-2, thereby rendering treated explants Wnt signaling-deficient, and found that distal/acinar development proceeded normally. Together, these data support a model wherein Wnt/β-catenin signaling primarily functions to drive maximal proliferation in both the early epithelium and in acinar cells, while proximal-distal patterning and acinar development rely on the structural role of β-catenin.

One potential caveat to this conclusion derives from the approach we have used to disrupt Wnt/β-catenin signaling. The *Ctnnb1*^{DM} allele has been thoroughly characterized in vitro and in vivo, and found to reduce Wnt/β-catenin signaling activity equivalently to a β-catenin null in both *Drosophila* and mammals [6]. Nonetheless, the βCAT^{DM} protein retains its TCF-binding domain and could potentially enter the nucleus and bind to these transcription factors. One of these, Tcf3/Tcf7L1, is thought to be regulated at the level of derepression rather than activation [32,33], and might be derepressed by βCAT^{DM} binding even if co-activators cannot by recruited. A mouse mutant of Tcf3 was recently developed that cannot bind β-catenin (*Tcf3*$^{\Delta N}$), and identifies phenotypes that depend on this derepressive mechanism [33]. We examined *Tcf3*$^{\Delta N/\Delta N}$ embryos and did not observe any pancreatic abnormalities, consistent with the lack of detectable *Tcf3* expression during pancreas development [9]. We therefore conclude that although βCAT^{DM} could still potentially bind to and derepress TCF factors, this mechanism is unlikely to compensate for loss of β-catenin-dependent activation in PBsKO pancreata. Nevertheless, future experiments are necessary to confirm that Wnt/β-catenin signaling is absent from the

pancreas in βCAT^{DM} animals.

The concept that β-catenin plays multiple, mechanistically-independent roles during organogenesis is not without precedent. In the neural tube, the signaling and structural roles of β-catenin perform separate but critical roles during development [6]. At a cell biological level, what might be the structural function of β-catenin in pancreas development? In our work investigating PBKO as well as acinar-specific β-catenin mutants (ABKO), we showed that despite the loss of β-catenin at the cell-cell junctions, E-cadherin localization appears normal [8,9]. We and others have shown that in the absence of β-catenin the desmosomal protein γ-catenin/plakoglobin is upregulated and localizes to E-cadherin+ adherens junctions, presumably acting redundantly to stabilize epithelial structures [9,23]. In this chapter, we demonstrated that the loss of β-catenin signaling did not induce upregulation or junctional localization of plakoglobin. We therefore assume that any yet-to-be-determined changes found in PBKO do not exist in PBsKO. As β-catenin links with the actin cytoskeleton via α-catenin, whether subtle, cytoskeletal changes occur when plakoglobin takes the place of β-catenin remains to be determined. Nevertheless, we are tempted to speculate that actin dynamics could be altered in PBKOs. The pancreas-specific deletion of the cytoskeletal regulator Cdc42 alters patterning, as evidenced by an increased number of undifferentiated MPCs caused by changes to polarity [11]. Such polarity defects led to failed ductal tube formation and increased epithelial-mesenchymal contact points, potentially enhancing MPC maintenance and acinar cell development [11,30].

We speculate that the loss of β-catenin at adherens junctions negatively affects actin dynamics, despite apparently normal E-cadherin localization, causing the observed

changes to patterning found in PBKO but not PBsKO. Moreover, determining that the signaling and structural functions of β-catenin are both necessary for separate aspects of pancreas development will stimulate further inquiry into other β-catenin loss-of-function phenotypes in which these two roles have not been distinguished. Finally, defining that pancreas development requires exact spatiotemporal control of both β-catenin functions could enhance future efforts to generate cell-based therapies for pancreatic diseases such as diabetes.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

The autoimmune destruction or dysfunction of pancreatic β-cells results in diabetes, which may be reversed at least transiently by the addition of new β-cells. Although functional β-cells can be obtained from cadaver donors, their availability is limited and insufficient to treat all insulin-dependent diabetics, and strategies to expand their numbers have met with only limited success to date. With their capacity for multilineage differentiation, pluripotent stem cells stand as a potentially inexhaustible source of β-cells; nonetheless, the milieu of signals and factors that drive β-cell development are not completely known. Exploiting the mechanisms controlling pancreatic organogenesis, and specifying progenitor lineages, will assist efforts to generate β-cells from stem cells for the purpose of treating diabetes. The work presented in this dissertation concentrates on the role of the multifunctional protein β-catenin during pancreas development, particularly during β-cell development. Therefore, the objectives of this dissertation were as follows: (1) To determine the role of β-catenin during β-cell development; (2) To elucidate the cellular mechanisms by which β-catenin controls the allocation of pancreatic lineages; (3) To distinguish if the Wnt signaling or structural function of β-catenin were required for pancreas development.

At the outset of my studies, the role of β-catenin during pancreas development was controversial [1]. Whereas some studies found that β-catenin was selectively required for exocrine acinar cell but not for endocrine β-cell development [2,3], others suggested that the Wnt signaling role of β-catenin was necessary for islet cell development [4]. These discordant results might be explained by several factors: First, the *Pdx1-Cre* deletor strains used to eliminate β-catenin were active either early, with no reported β-cell defects, or later with defects in islet cell number. Second, islet cell numbers were not accurately or thoroughly assessed in previous studies. Previously, β-cell mass was calculated by dividing the endocrine area by the total area of the pancreas, as measured across several representative sections. Such an approach, however, does not account for the loss of other pancreatic tissue such as acinar cells, potentially skewing the actual number of β-cells present [5]. Therefore, I developed a strategy to more accurately quantify the absolute mass or number of cell types in the pancreas, instead of their relative volume. The result of this strategy, presented in Chapter 2, was the confirmation of acinar cell and pancreatic hypoplasia and discovery of a more drastic reduction of β-cells than previously appreciated [6]. But why were fewer β-cells present, given that they appear to differentiate, function, and proliferate normally in the absence of β-catenin [2,3,7]? Where and when during development was β-catenin required to establish normal β-cell mass? By deleting β-catenin specifically from Ngn3+ islet precursors [8], which revealed no change in islet cell mass, I determined that β-catenin acts prior to endocrine specification to establish β-cell mass. Given these results, and because all pancreatic lineages arise from multipotent progenitor cells (MPCs) whose numbers determine organ

size [9-11], I hypothesized that the loss of β-cells in β-catenin deficient pancreata was due to the loss of MPCs.

By measuring pancreas size at various points during in utero development, I determined that the loss of β-catenin had little effect on pancreatic mass prior to E12.5. This finding is consistent with a previous study that reported pancreatic agenesis when β-catenin was ectopically activated prior to E12.5 [12], and suggests that the major role of β-catenin plays out after initial organ specification and bud formation. In fact, at E11.5 we found no difference in the number of MPCs present in PBKO and controls, providing evidence that the initial specification, patterning, and proliferation of MPCs occurs without β-catenin activity. However, just one day later at E12.5, the number of MPCs was dramatically reduced. Notably, the loss of MPCs from E11.5 to E12.5 coincided with an observed increase in Ngn3+ cells, indicating a failure to maintain distal-proximal patterning of the early organ, thus identifying a previously undescribed role for β-catenin during pancreas development. This early and specific loss of MPCs likely explains the specific loss of exocrine acinar cells reported by separate studies, as distal MPCs are eventually restricted to the acinar lineage [2,3,9]. The early proximalization of the β-catenin-deficient pancreatic epithelium occurred independent of impaired cell division that also occurred after E11.5 in both distal-proximal progenitor cells. This latter result may reflect the observed loss of the Wnt/β-catenin target gene *cMyc*, previously found to be necessary for robust pancreatic proliferation [13,14]. Therefore, we conclude that the defect in β-cell mass found at the end of development in PBKO was due to a combination of a shift in distal-proximal patterning and the reduced proliferation of progenitors, together leading to the selective loss of an effective progenitor pool.

Shifts in distal-proximal patterning, or changes to the allocation of pancreatic lineages are not restricted to β-catenin mutants. These have been seen in knockout studies of several pancreatic transcription factors, including the trunk determinant Nkx6.1 and the tip/acinar determinant Ptf1a [15]. Less is known about intercellular interactions that control distal-proximal patterning, although recent evidence implicates the Notch signaling pathway in this process. Initial studies of Notch signaling in the developing pancreas indicated that this pathway inhibits both endocrine and acinar cell differentiation [16-20]. More recent reports suggest that acinar inhibition by Notch could be due to effects on distal-proximal patterning, where Notch activity promotes proximal over distal fate as well as duct over endocrine fate [15,21,22]. Given our results indicating that β-catenin is required to maintain distal identity, and other reports suggesting that Notch inhibits distal fate in MPCs at roughly the same developmental stages, I hypothesized that the two pathways communicate to pattern the organ. A previous study used the γ-secretase inhibitor DBZ to inhibit Notch activity in pancreatic explants and found an expansion of the distal domain [22], suggesting that Notch activity promotes proximal fates even ex vivo. By culturing PBKO explants in the presence of DBZ, I was able to inhibit Notch signaling in the context of β-catenin deletion to test whether the two pathways interacted. In contrast to untreated PBKO explants, which developed very few β-catenin-deficient acinar cells, I found abundant β-catenin-deficient acinar cells in PBKO explants treated with DBZ. Therefore, inhibiting Notch signaling in the absence of β-catenin rescued distal patterning, potentially uncovering an antagonistic relationship between the two pathways, wherein β-catenin inhibits Notch activity. Cooperation between these two pathways is not without precedent, and has been extensively studied in

colorectal cancer [23]. Inhibitory crosstalk between β-catenin and Notch has not been described in the context of pancreas development.

How might β-catenin inhibit Notch signaling? One possibility might be through Dishevelled proteins, which are activated by both canonical and noncanonical Wnt ligands [24], and have been shown to block Notch signaling in the developing *Xenopus* epidermis through inhibition of the Notch transcription factor partner CSL/RBPJ [25]. However, β-catenin acts downstream of Dishevelled, and its deletion would not be predicted to prevent Dishevelled-CSL interactions. β-catenin could also negatively regulate the expression or localization of Notch signaling components and transcription factors, such that its deletion would result in overactivation of Notch to drive epithelial proximalization. Alternatively, β-catenin activity could directly or indirectly regulate the expression of Notch target genes such as Hes1, a known regulator of pancreas development [17,26]. Finally, the epistatic relationship proposed above does not exclude the possibility that these pathways act in parallel, and converge on a yet-unidentified downstream mechanism. Future experiments can distinguish between these models by analyzing the expression of Notch signaling components and target genes in the context of modified pancreatic β-catenin activity.

As a central participant in Wnt signaling and as a component of cadherin-based junctions, β-catenin is a multifunctional protein. However, the use of total β-catenin deletion in my studies of β-catenin in patterning and proliferation, described in Chapter 2, left open the question of whether these roles could be attributed to signaling or structural functions of β-catenin. Direct inhibition of the Wnt signaling pathway through the expression of a dominant-negative Frizzled8 Wnt receptor protein [27], or the deletion of

the Wnt target gene *cMyc* [13,14], reduced proliferation but did not clearly affect early patterning or the differentiation of MPCs into multiple pancreatic lineages. In light of these findings and my own from Chapter 2, we proposed that the canonical Wnt signaling role of β-catenin promotes proliferation and that the structural role of β-catenin could regulate patterning. However, proper genetic tools did not exist until recently to separate the signaling and structural functions of β-catenin. Through the utilization of a signaling-deficient, yet structurally competent allele of β-catenin (*Ctnnb1*^{DM}) [28], in Chapter 3, we addressed these two functions in pancreas development.

Wnt/β-catenin signaling-deficient pancreata (PBsKO) exhibited an obvious growth defect, appearing similar to PBKO at the end of development; however, further investigation revealed major differences in patterning and cell-differentiation between these models. As described previously, although some acinar cells develop in PBKO, these nearly all retain a functional copy of β-catenin, having "escaped" *Pdx1-Cre* recombination [2,3]. Conversely, less than half of all acini found in PBsKO were escapers, suggesting that acinar cell development and potentially pancreatic distal-proximal patterning occur independent of β-catenin signaling. Analyzing PBsKO, where β-catenin structural function had been "restored" compared to PBKO in which no β-catenin function is present, revealed a near 2-fold increase in the number of Ptf1a+ cells found at E12.5, indicating that distal-proximal patterning was partially rescued and maintained when the structural function of β-catenin was active. Moreover, proliferation was abrogated, but not entirely eliminated, in the absence of β-catenin signaling, both during early organogenesis and in postnatal acinar cells as shown in Chapter 3, explaining the loss of epithelial volume and indicating that Wnt/β-catenin signaling is required for

maximal but not absolute proliferation in the pancreas. Through the use of a mathematical model for pancreas growth, we demonstrated that even the modest change in proliferation found in PBsKO could account for the dramatic size discrepancy found later in development and adulthood.

Due to genetic redundancy and unknown cellular sources, whether Wnt ligands are actually required for pancreatic growth, or at some level for patterning, has not been addressed [1]. We eliminated all Wnt ligands from pancreatic buds, comprising pancreatic mesenchyme and epithelia by culturing E11.5 pancreatic buds in the presence of IWP-2[29], a potent inhibitor of the Wnt modifier PORCN, which is needed for the secretion and activity of all Wnt ligands [30]. The resulting explants contained both distal and proximal populations, further supporting our hypothesis that distal-proximal patterning depends not on canonical Wnt/β-catenin signaling, but more likely on structural functions of β-catenin. Nevertheless, Wnt/β-catenin signaling remains crucial for the maximal proliferation and growth of the developing organ. In Chapter 2, I demonstrated reduced expression in PBKO of the Wnt target gene *cMyc*, which itself is required for pancreatic progenitor and acinar cell proliferation but not maintenance [13]. Therefore, a model in which Wnt-dependent *cMyc* expression drives organ growth is plausible. However, the source of the Wnt ligands that drive pancreas growth throughout in utero development and in adulthood [7] cannot be determined through pharmaceutical inhibition as discussed above, and remains unknown.

At the onset of pancreas development, epithelial buds expand into pancreatic mesenchyme, which studies from the 1960s identified as a potential source of extrinsic signals that promote exocrine pancreas growth and differentiation [31,32]. Because the

obvious defects in acinar cell growth found in PBKO [2,3], PBsKO (Chapter 3), and *cMyc* mutants mimic those found after the removal of pancreatic mesenchyme, we speculate that mesenchymally-derived Wnts promote acinar cell proliferation. In addition, as discussed in the Appendix of this dissertation, the pancreatic epithelium may also serve as a potential source of Wnt ligands needed for maximal pancreas growth. Understanding the source and variety of Wnt signals that drive pancreatic progenitor expansion, independent of patterning or lineage specification, could enhance efforts to derive β-cells from pluripotent stem cells. Nonetheless, the mesenchyme must promote acinar development via Wnt-independent mechanisms as well, given that acinar cells still develop in cultured PBsKO explants but not mesenchyme-deficient explants [31,32]. It will be interesting to determine if the primary effect of these mesenchymal signals is on proximal-distal patterning, potentially modulated by β-catenin structural function.

The notion that the structural and signaling functions of β-catenin exert independent and important effects on organ development was first shown, using *Ctnnb1*^{DM}, in the developing spinal cord [28]. However, what the structural role of β-catenin might be during pancreas development remains elusive. No defects in E-cadherin localization were observed occurred following deletion of β-catenin in the pancreas [3,7]. This is likely the result of the upregulation and redundant activity of the desmosomal protein γ-catenin/plakoglobin localizing to E-cadherin junctions [3,33]. Such upregulation was confirmed in PBKO but not PBsKO samples, leading us to infer that structural integrity remains intact in PBsKO. It is tempting to speculate that the association of plakoglobin with E-cadherin could potentially destabilize desmosomal junctions, which have been shown to regulate growth and branching in mammary cells, [34] and thereby

partially explain the discrepancy between PBKO and PBsKO. Nevertheless, the actin cytoskeleton is linked to E-cadherin through β-catenin, and whether any cytoskeletal changes occur in the absence of β-catenin has not been determined.

Several revealing studies indicate that actin dynamics and cellular polarity are crucial for the maintenance of distal-proximal patterning. Deleting the cytoskeletal regulator Cdc42 from the developing pancreas alters epithelial tube formation by trunk cells, and causes increased epithelial-mesenchymal contacts, an interaction that is likely crucial for acinar cell development and MPC maintenance [31,35]. Furthermore, other studies showed that changes to polarity, coordinated in part by the Rho GTPase-activating protein (Rho-GAP) Stard13 and the EphB2/B3 receptors, are needed to generate and maintain the distal-proximal pattern of the early pancreas, and that disruptions to epithelial polarization adversely affect pancreas development [36,37]. How could changes in actin reorganization lead to changes in distal-proximal patterning? One possibility is that alterations of the cytoskeleton could affect localization or activity of Notch ligands or receptors, producing patterning abnormalities such as enriched distal patterning observed following Notch inhibition [21,22]. Characterization of the cytoskeletal changes present in PBKO but not PBsKO will provide a basis for future investigation of this important problem.

Together, the data presented in the preceding chapters of this dissertation provide a new model for the function of β-catenin during pancreas development (Fig. 4.1). We found that β-catenin is required to establish β-cell mass by maintaining distal-proximal patterning, and by expanding the progenitor pool. In so doing, we clarify previously conflicting accounts of the role of β-catenin during β-cell development [1]. Furthermore,

we established that pancreatic specification and initial outgrowth occur independent of β-catenin activity. Nevertheless, a transition to β-catenin dependence, the basis for which is not known, occurs simultaneously with morphological changes to the pancreatic epithelium that establish distal-proximal domains [10]. We speculate that once fate is established, β-catenin is no longer required to maintain cellular identity, instead functioning primarily to drive proliferation. In addition, for the first time, the signaling and structural functions of β-catenin during pancreas development have been separated and assigned pro-growth and patterning roles, respectively. Therefore, precise spatiotemporal control of both the signaling and structural function of β-catenin is crucial to the development of all pancreatic lineages.

Future attempts to generate functional β-cells from pluripotent stem cells in vitro will require a deeper understanding of the extrinsic and intrinsic mechanisms that govern pancreas development. The information presented in this dissertation proposes that the time-appropriate addition of Wnt ligands, and the potential small molecule manipulation of β-catenin-mediated structural functions, could be key to the expansion and cell fate determination of stem cell-derived pancreatic progenitors, respectively. Consequently, programming β-cell fate will benefit from these and future studies of the mechanisms through which β-catenin, and other related molecules, control pancreas development.

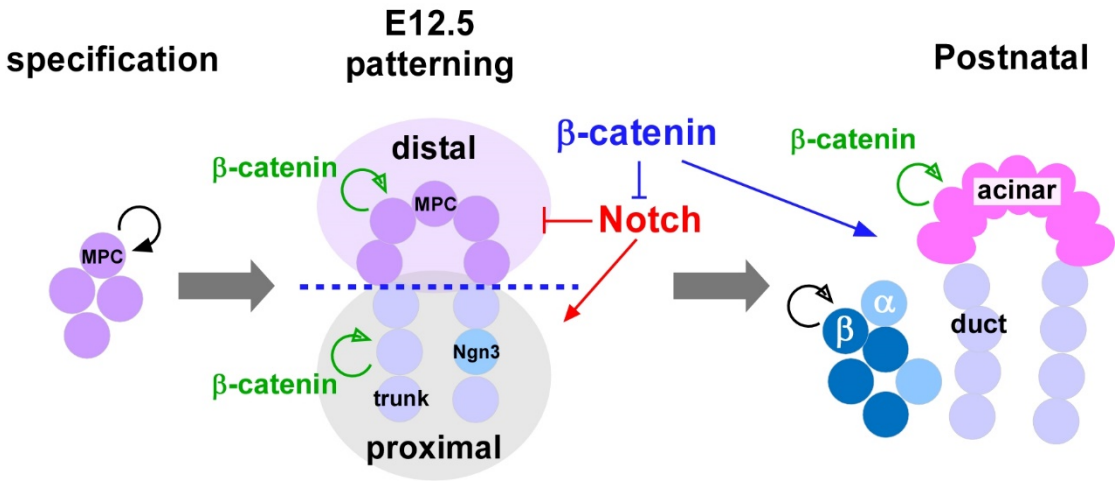

Figure 4.1: The roles of β-catenin during pancreas development. Following specification and prior to E12.5, MPCs (purple) are relatively unorganized, until a dramatic shift in morphology occurs referred to here as patterning. After E12.5, MPCs are found at the distal-tip of the pancreatic epithelium (shaded purple) and proximal-trunks (shaded grey) comprise trunk progenitors and Ngn3+ endocrine precursors. A new role for β-catenin in maintaining the distal-proximal patterning of early pancreatic progenitors has been identified, indicated by the blue dashed line between the two domains at E12.5. Evidence suggests that the structural role of β-catenin (blue lettering/arrows) mediates this process and we hypothesize that this is performed, in part, through negative regulation of Notch signaling. The signaling role of β-catenin (green lettering/arrows) is required for pancreatic growth and proliferation of the distal and proximal cells during early phases of development and during acinar postnatal acinar cell expansion. Black arrows indicate proliferation occurring independent of β-catenin.

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APPENDIX

WNT/β-CATENIN SIGNALING INHIBITS ADIPOGENESIS IN THE ADULT PANCREAS

Generated in collaboration with Rachel Redman Hulse, M.S.

Results

Previous studies, along with the data presented in this thesis, indicate that the canonical Wnt/β-catenin signaling pathway directs various aspects of pancreas development [1-3]. As the majority of these studies manipulated Wnt signaling by mutating signaling components in the Wnt receiving cells, direct evidence for Wnt ligand activity in the pancreas has not been determined. Moreover, genetic redundancy amongst Wnt ligands prevents straightforward examination of individual Wnts and complicates studies aimed at identifying potential sources of Wnt signals. However, all Wnt ligand secretion and activity requires the function of a single endoplasmic reticulum-localized acyltransferase enzyme Porcupine/Porcn [4]. Recently, the Murtaugh lab generated a conditional *Porcn* knockout allele, deletion of which prevents Wnt secretion [5], providing a tool to assay ligand-dependent aspects of Wnt signaling.

To determine the role of epithelial derived Wnts during pancreatic organogenesis, we deleted *Porcn*^{lox} [5] from the early pancreatic progenitors with *Pdx1-Cre* [6] to create pancreas specific *Porcn* knockouts (PPKO). In contrast to β-catenin mutants, casual observation revealed no obvious defect in the pancreatic epithelium near birth (data not shown), indicating that epithelial-derived Wnts are not necessary for pancreas development in utero. In addition to its developmental role, β-catenin activity is also required for acinar cell expansion following birth [1]; nevertheless, whether epithelial-derived Wnts were required for postnatal pancreas growth and homeostasis was not known. Our analysis of adult PPKO mice revealed that their pancreata were reduced in mass compared to sibling controls (data not shown). However, in histological analysis of 3-month-old PPKO mice, we unexpectedly discovered the presence of numerous adipocytes located within exocrine pancreas lobules. Intrapancreatic adipocytes were even more abundant, apparently replacing acinar cells, at 6 months of age, but were not detectable in 1-month-old PPKO mice (Fig. A.1A-F).

To determine when adipocytes form within the adult pancreas, we examined PPKO mice between 3 weeks and 6 months of age. We found that intrapancreatic adipocytes were not present prior to 2 months of age, but increased progressively from 2 to 6 months to occupy >50% of the exocrine pancreas (Fig. A.1G). Many studies have shown that Wnt signaling inhibits adipogenesis in vitro [7], and these data provide evidence that Wnts from the pancreatic epithelium prevent adipogenesis in the adult pancreas. However, which cell type receives Wnt signals to prevent such adipogenesis could not be determined in PPKO, as Wnts could have both autocrine and paracrine effects.

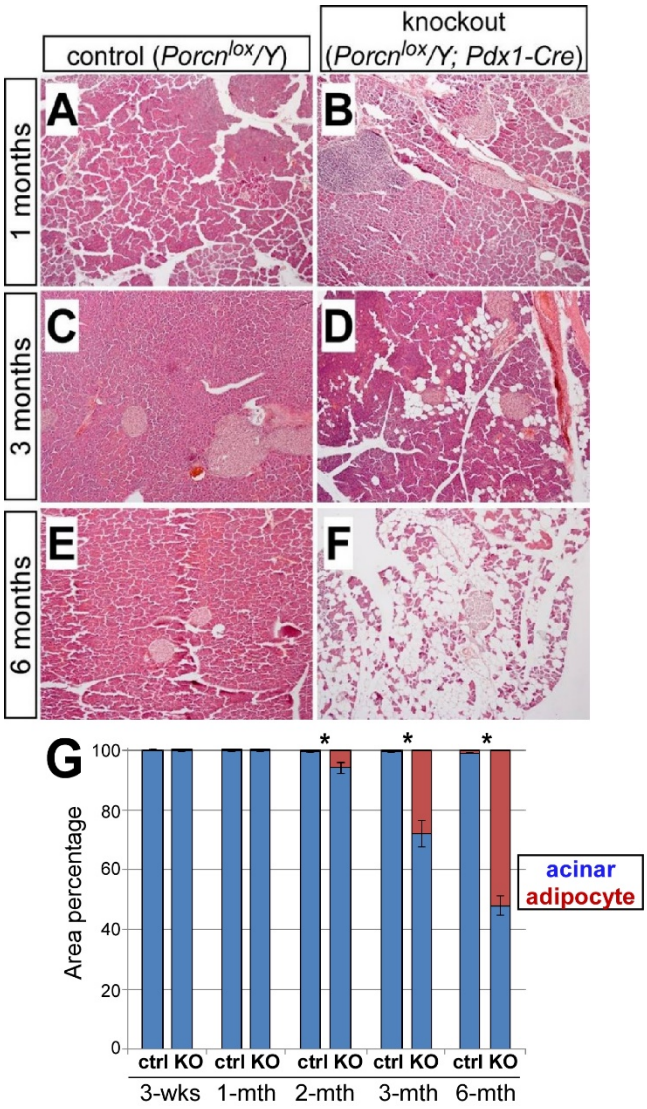

Figure A.1. Adipocytes form in adult *Porcn* deficient pancreata. Pancreas-specific *Porcn* knockouts (PPKO) were harvested at several points after birth, stained by H&E, and the percentage of adipocytes quantified. (A-B) One-month-old control and PPKO pancreata did not contain intrapancreatic adipocytes. (C-D) Adipocytes are present in 3-month-old PPKO pancreata but not controls. (E-F) At six months, entire lobules of pancreatic tissue are inundated with adipocytes in PPKOs. (G) Quantification of the percentage of pancreatic area containing normal acinar cells (blue portion of bar) or adipocytes (red portion of bars) from 3 weeks through 6 months of age reveals an increasing trend of adipogenesis in the adult PPKO pancreas.

In Chapter 3, we found that acinar cells develop in the absence of Wnt/β-catenin signaling, but not in the absence of total β-catenin function [2,8]. Therefore, Wnt signaling-deficient acinar cells provided a new opportunity to test whether acinar-autonomous Wnt signaling suppresses adipogenesis, arising either from epithelial transdifferentiation or adipocyte differentiation by some other nonepithelial cell type. We harvested β-catenin signaling-deficient pancreata (PBsKO) from 4-5-month-old mice and, unexpectedly, found adipocytes developing within the exocrine domain of β-catenin-negative lobules (Fig. A.2A-F). This phenotype was strikingly similar to that observed in PPKO mice of similar age. Moreover, in a separate study, pancreas-specific deletion of c-Myc, a known Wnt target gene, also resulted in the appearance of adipocytes[9], supporting the notion that Wnt signaling within pancreatic epithelial cells is necessary to suppress adipogenesis. It remains unknown whether these adipocytes arose from acinar cells, which would constitute an unprecedented endoderm-to-mesoderm cell fate switch, or from non-epithelial cells (e.g., pancreatic stellate cells), whose differentiation was prevented non-cell-autonomously by Wnt/β-catenin signaling within adjacent epithelial cells. Importantly, lineage tracing of adipocytes in *Porcn* mutant pancreata (PPKO) indicated that they arose from nonepithelial cells, supporting the latter mechanism (R. Hulse, unpublished results). Future experiments will utilize lineage tracing to determine the cellular origin of these adipocytes developing in PBsKO pancreata. In any event, although the physiological or metabolic impact of intrapancreatic adipocytes is not known, eliminating Wnt/β-catenin signaling from the pancreatic epithelium could serve as model to study this phenomenon.

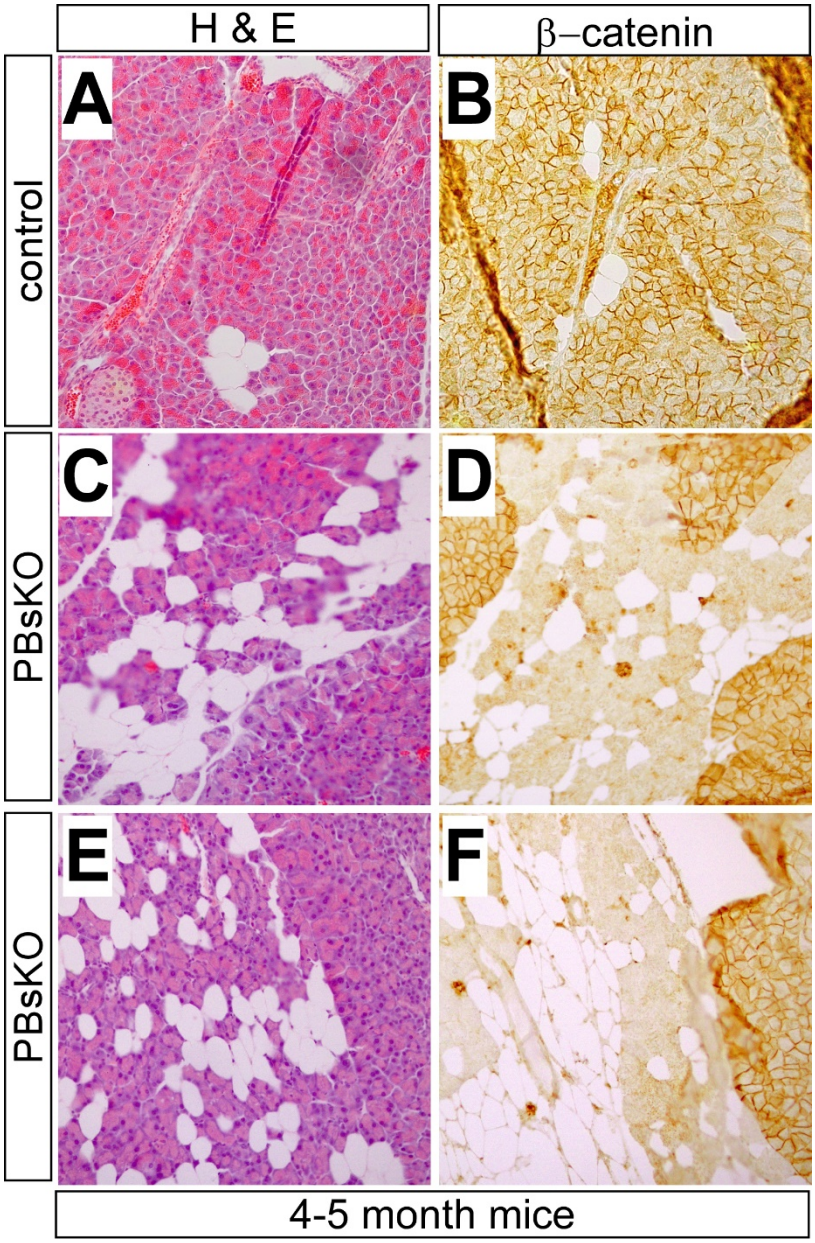

Figure A.2. Intrapancreatic adipocytes form in the absence of β-catenin signaling function. Mice 4-5 months of age were harvested and stained histologically to identify adipocytes and adjacent sections stained for β-catenin to distinguish β-catenin- mutant cells from β-catenin+ escapers. (A-B) Rare adipocytes are found in β-catenin+ adult pancreata. (C-F) Fields from two separate 4-5-month-old PBsKO pancreata showing adipogenesis within the pancreatic epithelium. (D, F) Adjacent sections from C and E, respectively, stained for β-catenin that adipocytes are only found in β-catenin deficient lobules.

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